ADULT-TYPE HYPOLACTASIA:
Genotype-phenotype correlation

Heli Rasinperä

Department of Medical Genetics, Faculty of Medicine, University of Helsinki,
and
Hospital for Children and Adolescents, University of Helsinki, Finland

Academic Dissertation

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Supervised by:

Docent Irma Järvelä, M.D.,Ph.D. and **Docent Kaija-Leena Kolho, M.D., Ph.D.**
Department of Medical Genetics
University of Helsinki
Finland

Hospital for Children and Adolescents,
University of Helsinki
Finland

Reviewed by:

Docent Riitta Korpela, Ph.D. and **Professor Ann-Christine Syvänen, Ph.D.**
Institute of Biomedicine,
Pharmacology
University of Helsinki
Finland

Department of Medical Sciences
Uppsala University
Sweden

To be publicly discussed with:

Professor Olli Simell, M.D., Ph.D.
Department of Pediatrics
University of Turku
Finland

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II **Rasinperä H**, Kuokkanen M, Kolho K-L, Lindahl H, Enattah NS, Savilahti E, Orpana A, Järvelä I: Transcriptional downregulation of the lactase (LCT) gene during childhood. Gut 2005; 54:1660-1.

III **Rasinperä H**, Saarinen K, Pelkonen A, Järvelä I, Savilahti E, Kolho K-L: Molecularly defined adult-type hypolactasia in school-aged children with a previous history of cow's milk allergy. World J Gastroenterol 2006; 12:2264-8.

IV Tikkakoski S, **Rasinperä H**, Kotamies A, Komu H, Pihlajamäki H, Kolho K-L, Järvelä I: Molecularly defined adult-type hypolactasia among working age people with reference to milk consumption and gastrointestinal symptoms. Submitted.

V **Rasinperä H**, Forsblom C, Enattah NS, Halonen P, Salo K, Victorzon M, Mecklin J-P, Järvinen H, Enholm S, Sellick G, Alazzouzi H, Houlston R, Robinson J, Groop P-H, Tomlinson I, Schwartz S Jr, Aaltonen LA, Järvelä I, FinnDiane Study Group: The C/C_{-13910} genotype of adult-type hypolactasia is associated with an increased risk of colorectal cancer in the Finnish population. Gut 2005; 54:643-7.

ABBREVIATIONS

aa	amino acid
AMV	avian myeloblastosis virus
BHT	breath hydrogen test
bp	base pair
cDNA	complementary deoxyribonucleic acid
Cdx-2	caudal-type homeobox transcription factor 2
CLD	congenital lactase deficiency
CMA	cow's milk allergy
CMSE	cow's milk protein-sensitive enteropathy
COS	African-green-monkey kidney cell
cpm	counts per minute
CRC	colorectal carcinoma
cSNP	coding single nucleotide polymorphism
DARS	aspartyl-transfer ribonucleic acid synthetase
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotidetriphosphate
DTT	dithiothreitol
EMSA	electromobility shift assay
ER	endoplasmic reticulum
FREAC	forkhead-related activator
GADPH	glyceraldehyde-3-phosphate
GLUT5	glucose transporter 5
HNF1α	hepatic nuclear factor 1α
HOXC11	homeo-box C11
IBS	irritable bowel syndrome
kb	kilobase
LD	linkage disequilibrium
LM	lactose malabsorption
LPH	lactase-phlorizin hydrolase
LTT	lactose tolerance test
LTTE	lactose tolerance test with ethanol
Mb	megabase
MCM6	minichromosome maintenance 6
MMR	mismatch repair
mRNA	messenger ribonucleic acid
NSAID	non-steroidal anti-inflammatory drug
p	short arm of chromosome
PCR	polymerase chain reaction
PSP	phenolsulphthalein
q	long arm of chromosome
RAP	recurrent abdominal pain
REHH	relative extended haplotype homozygosity
RNA	ribonucleic acid
RT	reverse transcriptase
RT-PCR	reverse transcriptase polymerase chain reaction
SCFA	short-chain fatty acid
SGLT1	sodium-dependent glucose transporter 1
SNP	single nucleotide polymorphism

SUMMARY

Adult-type hypolactasia (primary lactose malabsorption), is the most common enzyme deficiency in humans, and present in majority of the world's population. In Finland 18% of the population have adult-type hypolactasia, and majority of them have symptoms (lactose intolerance) weekly. Adult-type hypolactasia has been shown to explain approximately one third of unspecific abdominal complaints. Adult-type hypolactasia is genetically determined and manifests during childhood when lactase activity declines to about 10-15% of the activity at birth. Adult-type hypolactasia is a common phenomenon for all sucklings. A pup is weaned from milk when it can manage without its mother's milk, and lactase enzyme activity disappears from its intestine.

A mutation that allows the persistence of lactase activity in the intestine occurred in man thousands of years ago. Our research group identified a genetic variation associated with adult-type hypolactasia, a one base polymorphism C>T_{-13910} year in 2002. This polymorphism is located approximately 14 kilobases from the starting codon of the lactase-phlorizin hydrolase (LPH) gene, in intron 13 of the minichromosome maintenance 6 (MCM6) gene in chromosome 2q21-22. The variant is inherited recessively so that the C_{-13910} allele in a homozygous form (the C/C_{-13910} genotype) is always associated with adult-type hypolactasia and the T_{-13910} allele (C/T_{-13910} and T/T_{-13910} genotypes) with persistence of lactase activity. The C/T_{-13910} polymorphism has been shown to be associated to the regulation of lactase enzyme activity at the transcriptional level.

In this thesis, the timing and mechanism of decline of lactase enzyme activity during development was studied using the C/T_{-13910} variant associated with adult-type hypolactasia as a molecular marker. We observed that the C/C_{-13910} genotype associated with low lactase activity in all children aged > 12 years, despite their ethnicities. Lactase activity declined in Finnish children at an age of five to 12 years, in other Caucasians between three to six years and in children of African origin before the age of eight years. The sensitivity of the genetic test of adult-type hypolactasia in those aged > 12 years was 93% and the specificity 100%. In addition, we noticed that

the relative expression of lactase mRNA from the C_{-13910} and T_{-13910} alleles was equal in children up to four years of age. At ages between four and five years, the expression of lactase mRNA from the C_{-13910} allele began to decline in comparison to the T_{-13910} allele. In children aged > six years, the lactase mRNA expression from the C_{-13910} allele had declined to < 20% of that from the T_{-13910} allele. Thus, we showed that the relative expression of lactase mRNA from the C_{-13910} allele associated with adult-type hypolactasia declines during development and associates with the decline of lactase enzyme activity in subjects with the homozygous C/C_{-13910} genotype.

In this work we also studied the relation of milk consumption and the milk-related abdominal complaints to the C/T_{-13910} genotypes associated with lactase persistence/non-persistence. We noticed that children and adults with the C/C_{-13910} genotype associated with low lactase activity consumed significantly less dairy products compared to those with the C/T_{-13910} or T/T_{-13910} genotypes. Flatulence was the only of the classic symptoms of lactose intolerance that subjects with genotype C/C_{-13910} reported significantly more often than those with the C/T_{-13910} or T/T_{-13910} genotypes. There was no association between cow's milk allergy and adult-type hypolactasia.

Furthermore, in this study we examined the association of adult-type hypolactasia and colorectal carcinoma. Our results showed a significant association between the C/C_{-13910} genotype associated with low lactase activity and an increased risk of colorectal carcinoma. Any association between the age of the subjects, site, histology or degree of the tumor could not, however, be detected. No association between the C/C_{-13910} genotype and colorectal carcinoma was seen in British and Spanish populations. Further studies are needed to clarify the role of dairy products in colorectal carcinoma among the C/T_{-13910} genotype groups.

TIIVISTELMÄ

Primaari maitosokerin eli laktoosin imeytymishäiriö on ihmisen yleisin entsyyminpuutos, ja sitä esiintyy suurimmalla osalla maapallon väestöstä. Suomessa 18%:lla väestöstä on laktoosin imeytymishäiriö ja heistä enemmistö oireilee viikottain (laktoosi-intoleranssi). Laktoosin imeytymishäiriön on todettu selittävän noin kolmasosan epämääräisistä vatsavaivoista. Laktaasi-entsyymin aktiivisuuden aleneminen lapsuuden aikana suolen limakalvolla 10-15%:iin syntymähetkellä tavattavasta aktiivisuudesta on perinnöllisesti määräytynyt ominaisuus. Laktoosin imeytymishäiriö on kaikille nisäkkäille tyypillinen ilmiö. Pennun tullessa toimeen ilman emon maitoa, on tarkoituksenmukaista vieroittaa pentu rinnasta ja laktaasi-entsyymin aktiivisuus häviää imeväisen suolistosta.

Ihmiselle on vuosituhansia sitten tapahtunut mutaatio, joka sallii laktaasin aktiivisuuden säilymisen suolessa. Tutkimusryhmämme tunnisti laktoosin imeytymishäiriöön liittyvän geenimuutoksen, yhden emäksen polymorfian C>T $_{-13910}$ vuonna 2002. Muutos sijaitsee n. 14 kiloemäksen päässä lactase-phlorizin hydrolase (LPH) -geenin aloituskodonista minichromosome maintenance 6 (MCM6) -geenin intronissa 13 kromosomissa 2q21-22. Kyseinen muutos osallistuu laktaasin säätelyyn transkriptiotasolla. Se periytyy peittyvästi siten, että C/C$_{-13910}$ -genotyyppi liittyy aina laktoosin imeytymishäiriöön ja C/T$_{-13910}$ tai T/T$_{-13910}$ -genotyypit laktoosin sietoon.

Väitöskirjatyössäni tutkin normaaliin kehitykseen kuuluvan laktaasi-entsyymin aktiivisuuden laskun yhteyttä äskettäin tunnistettuun MCM6 -geenin intronissa sijaitsevaan C/T$_{-13910}$ -emäsmuutokseen sekä laktaasi-aktiivisuuden muutoksen mekanismia lapsuusiällä. Töissämme totesimme C/C$_{-13910}$ -genotyypin assosioituvan matalaan laktaasi-aktiivisuuteen kaikilla yli 12-vuotiailla lapsilla etnisyydestä riippumatta. Suomalaislapsilla laktaasin aktiivisuus aleni viiden-12 vuoden iässä, muilla valkoisen rodun edustajilla kolmesta kuuteen -vuotiaina ja afrikkalaisilla ennen kahdeksan vuoden ikää. Laktoosin imeytymishäiriön geenitestin sensitiivisyys oli 93% ja spesifisyys 100% yli 12-vuotiailla. Totesimme myös, että suhteellisesti yhtä paljon laktaasi mRNA:ta tuotetaan sekä C$_{-13910}$ että T$_{-13910}$ -alleelista lapsilla neljän vuoden ikään saakka. Neljän ja viiden ikävuoden välillä laktaasi-mRNA:n tuotto C-

$_{13910}$ -alleelista alkoi laskea T$_{-13910}$ -alleeliin verrattuna, ja yli kuusi-vuotiailla se oli vähentynyt < 20%:iin T$_{-13910}$ -alleelin ekspressiosta. Näin ollen, osoitimme laktoosin imeytymishäiriöön assosioituvan C$_{-13910}$ -alleelin ekspression vähenevän iän myötä ja osallistuvan kehityksen aikana tapahtuvan laktaasi-aktiivisuuden laskuun mRNA-tasolla henkilöillä, joiden genotyyppi on homotsygootti C/C$_{-13910}$.

Tutkimuksessa selvitimme myös maidon käytön ja maidon aiheuttamien oireiden yhteyttä laktoosin imeytymishäiriön perintötekijätyyppeihin suomalaislapsilla ja -aikuisilla. Totesimme sekä lasten että aikuisten, joilla on matalaan laktaasi-aktiivisuuteen assosioituva C/C$_{-13910}$ -genotyyppi, käyttävän merkitsevästi vähemmän maitotuotteita kuin genotyyppiryhmiin C/T$_{-13910}$ ja T/T$_{-13910}$ kuuluvat. Klassisista laktoosi-intoleranssin oireista vain ilmavaivat olivat merkitsevästi yleisempiä laktoosin imeytymishäiriöstä kärsivillä. Maitoallergian ja laktoosi-intoleranssin välillä emme todenneet yhteyttä.

Lisäksi väitöskirjassani selvitimme onko laktoosin imeytymishäiriöllä ja sen mahdollisesti aiheuttamalla suolen ärsytyksellä yhteyttä paksunsuolensyövän syntyyn. Totesimme matalan laktaasi-aktiivisuuden määrittävän C/C$_{-13910}$ genotyypin liittyvän suomalaisilla merkitsevästi kohonneeseen paksunsuolensyövän riskiin. C/C$_{-13910}$ genotyypin yliesiintyminen ei liittynyt ikään, kasvaimen paikkaan, histologiaan tai syövän asteeseen. Englantilaisilla ja espanjalaisilla emme todenneet yhteyttä paksunsuolensyövän ja C/C$_{-13910}$ -genotyypin osalta. Lisätutkimuksissa pyrimme selvittämään maitotuotteiden käytön merkitystä paksunsuolensyövässä eri laktoosin imeytymishäiriön perintötekijätyyppi-ryhmissä.

INTRODUCTION

Milk is an excellent source of the nutrients needed for a healthy diet. It is especially important for newborns, as it is their primary energy source. The milk sugar, lactose, is a disaccharide composed of galactose and glucose units. Lactose cannot be utilized as such but needs first to be hydrolyzed by the enzyme lactase-phlorizin hydrolase (LPH) in the brush-border membrane of the small intestinal epithelium to its constituent monosaccharides, which are quickly absorbed (1, 2). Lactase has a unique pattern of activity in animals: its level in mammals increases shortly before birth and remains high until weaning. After that, lactase is downregulated and its activity declines to the very low levels seen in adult mammals (adult-type hypolactasia). In humans, a considerable number of individuals maintain high levels of intestinal lactase activity throughout adulthood, whereas in the rest lactase activity declines to about one-tenth of the activity of infants (2).

The majority of subjects with low lactase activity experience symptoms of lactose intolerance such as flatulence, bloating and diarrhea after consumption of lactose containing milk products; the symptoms, however, vary widely among individuals (3). Although the symptoms are most often mild, adult-type hypolactasia has a significant role in unspecific abdominal complaints in populations with high consumption of dairy products. The diagnosis of adult-type hypolactasia is usually based on the lactose tolerance test (LTT), which measures the increase in blood glucose after an oral lactose load (4). The incidence of false-positive results in LTT, however, is high; in children it may even be as high as 30% (5). The assay of mucosal disaccharidases directly from an intestinal biopsy sample is the reference method, although it is unsuitable for everyday clinical practice (4, 6).

Adult-type hypolactasia, i.e., a low lactase activity in the intestine was in 1973 shown to be inherited as an autosomal recessive trait (7). In 2002, a single-nucleotide polymorphism (SNP), C to T change 13,910 basepairs (bp) from the 5'end of lactase (LCT) gene trait was identified to be associated with the lactase persistence/non-persistence trait. The homozygous C/C_{-13910} genotype completely associated with low lactase activity in the small intestine (8). This finding has allowed functional studies

of the variant in regulation of the LCT gene, population genetic studies as well as studies on the association of the variant in various diseases to be carried out. Here the studies were focused on the timing and mechanism of downregulation of lactase activity in the intestine of children at various ages and ethnicities at the protein, DNA and RNA levels (I, II). Moreover, this study has assessed the applicability of the genetic test of the C/T_{-13910} variant as a screening method for adult-type hypolactasia as a cause of gastrointestinal symptoms in children and in Finnish adults (I, III, IV). The consumption of milk products, and the prevalence of various gastrointestinal symptoms have been studied in Finnish children and adults, and the findings have been related to the C/T_{-13910} genotype of the subjects (I, III, IV). Finally, the identification of the C/T_{-13910} variant has allowed us for the first time to conduct a large-scale study on the role of lactase activity as a risk factor on the development of colorectal carcinoma in three different populations (V).

REVIEW OF THE LITERATURE

1. LACTOSE AND ITS METABOLISM

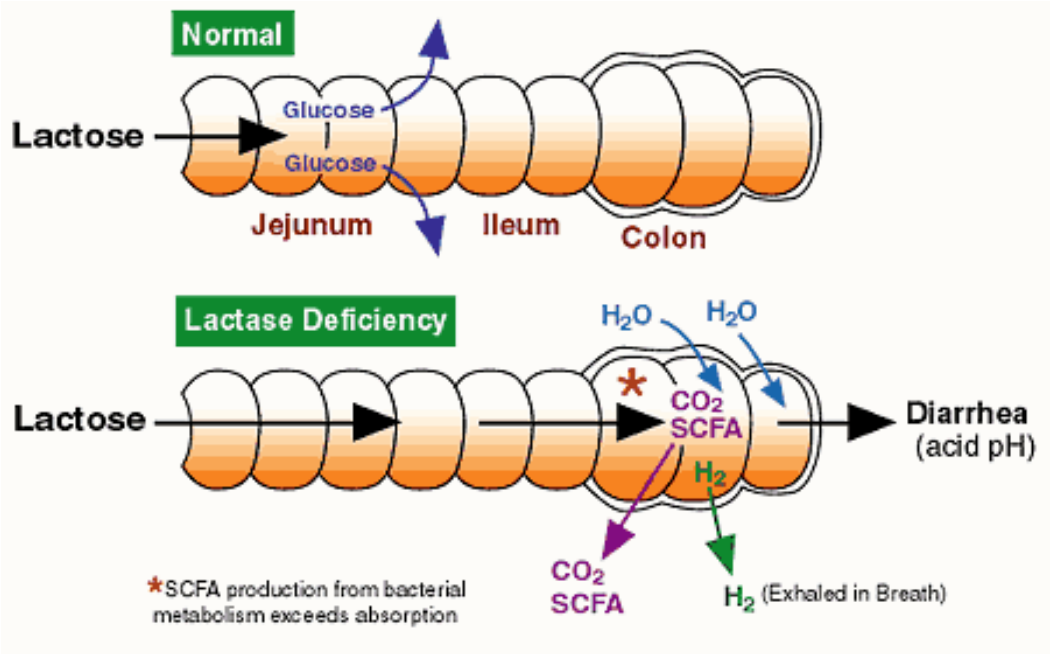
Lactose, the main carbohydrate of milk, is synthesized in the mammary gland by participation of β1,4 galactosyl transferase (EC 2.4.1.22) and α-lactalbumin (9-11). The concentration of lactose in milk varies between species (12). Cow's milk contains 4.7 g lactose in 100 ml of milk, and human milk, in which the lactose content is highest of all mammals, 7.0 g in 100 ml (13). Lactose is the primary energy source of newborns. Lactose has several applications in the food industry because of its physiological properties: it provides good texture and binds water as well as color. It is less than half as sweet as glucose (14).

Lactose is a disaccharide composed of galactose and glucose units joined by a β(1-4) linkage. After ingestion of lactose, it is hydrolyzed into glucose and galactose monosaccharides by lactase-phlorizin hydrolase (LPH) enzyme in the brush-border membrane of the small intestinal epithelium. These monosaccharides are transported through the intestinal epithelium using the sodium-dependent glucose transporter 1 (SGLT1) (15). Glucose enters the body glucose pool directly, whereas galactose is first metabolized to glucose by UDP-galactose 4-epimerase. This occurs mainly in the liver and is extremely efficient (16).

In subjects with low levels of lactase activity, most of lactose remains unhydrolyzed in the jejunum. The osmotic load of the unhydrolyzed lactose in the small intestine results in an influx of water into the lumen, which contributes to rapid intestinal transit (Figure 1). In the colon, microbes metabolize lactose into various gases and short-chain fatty acids (SCFAs). Carbon dioxide, hydrogen and methane are the principal gases formed and in excess they cause abdominal distension, bloating and flatulence. The gases diffuse into the blood stream and are exhaled through the lungs or excreted as flatus (1). The SCFAs formed include among others acetic, butyric, propionic, succinic, lactic and formic acids (17). The SCFAs are rapidly absorbed from the intestine. Acetic acid, which represents up to 50% of the total SCFAs is finally metabolized in the peripheral tissues, whereas butyric and propionic acids undergo final metabolism in the liver. The individual differences in the composition

of intestinal microbiota have an effect on the rate of lactose fermentation and thus account for the different tolerance to lactose in subjects with adult-type hypolactasia (1).

Figure 1. Metabolism of lactose in subjects with high and low lactase activities. (From: UCLA Center for Human Nutrition).



2. LACTASE-PHLORIZIN HYDROLASE (LPH)

Lactase-phlorizin hydrolase (LPH) was in 1906 shown to be present in the intestine of pups (18, 19). After half a century, entire curves of lactase enzymatic activity during development had been constructed for several animal species (20, 21). Holzel, in 1959, was the first to describe children with intolerance to lactose (22). Adult-type hypolactasia was reported by Italian and Swedish groups in 1963 (23, 24). The gene encoding lactase-phlorizin hydrolase was localized to chromosome 2q in year 1988 (25), and more specifically to 2q21 in 1993 (26). The complete primary structure of human LPH was established in 1988 (27) and the complete intron-exon organization in 1991 (28).

2.1 Structure

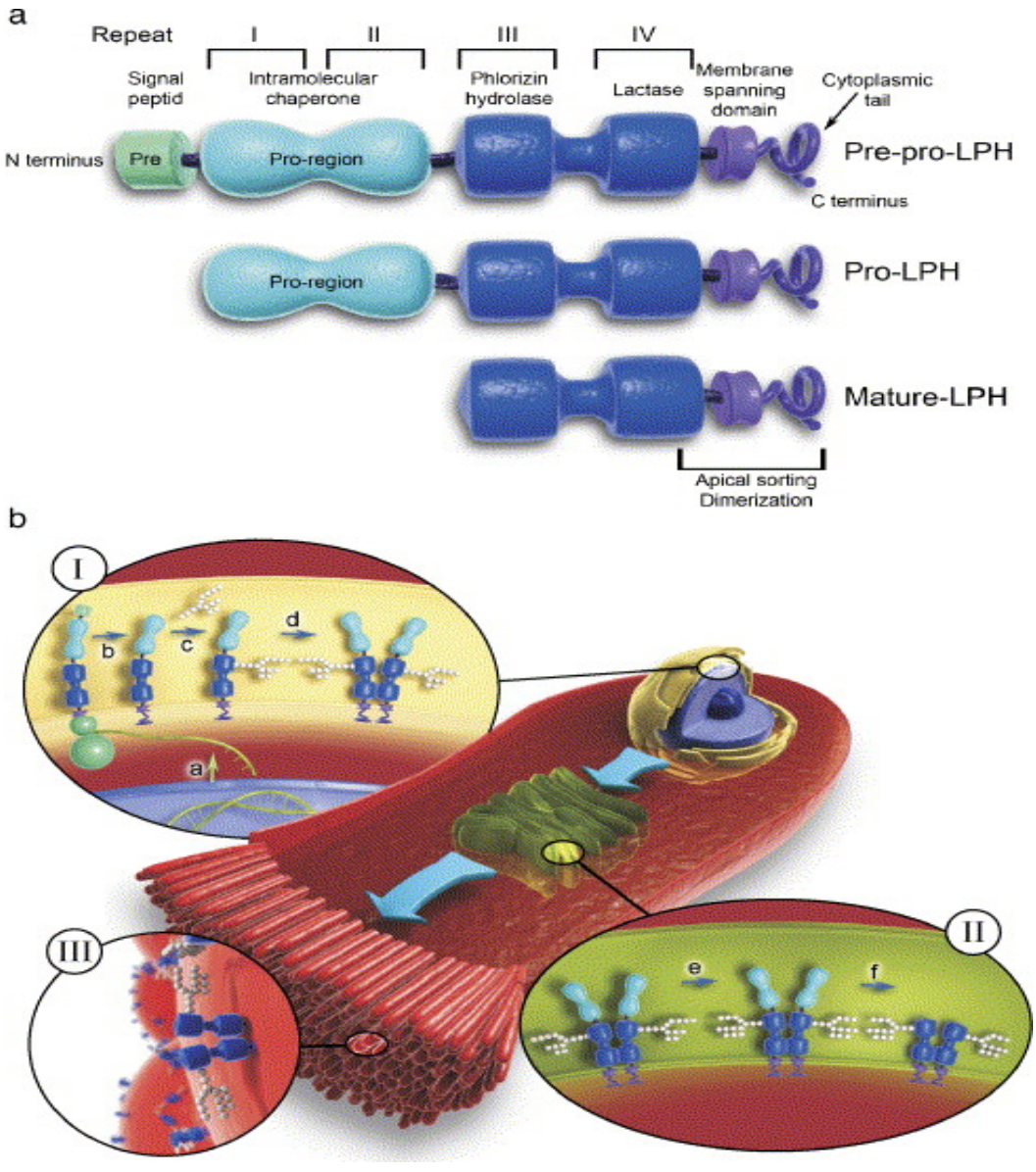
The human lactase-phlorizin hydrolase -gene (LCT) is about 55 kB in size and is composed of 17 exons (28). It encodes for lactase-phlorizin hydrolase (EC 3.2.1.23; 3.2.1.62), which is a β-galactosidase present most abundantly in the proximal jejunum. LPH has two activities: lactase hydrolyses lactose to galactose and glucose, whereas phlorizin hydrolase splits aryl- and alkyl-β-glycosides to phlorizin and β-glycosylceramides (2). In addition, the lactase activity cleaves cellobiose, cellotriose, cellotetrose and cellulose to a certain extent (29, 30). In spite of its two separate activities, LPH is synthesized as a single messenger RNA (mRNA) of 6247 bases (28), and translated as a pre-pro-polypeptide consisting of 1927 amino acids (aa) (27). There are four repeats in the LPH amino acid sequence, all homologous to a β-glycosidase unit, indicating that the gene has undergone two rounds of partial gene duplications (27). Because the phlorizin hydrolase activity is found in all vertebrates so far studied, but lactase activity is confined to mammals, it is believed that phlorizin hydrolase is the phylogenetic progenitor of both catalytic activities in the LPH complex (27). The human pre-pro LPH comprises five domains: (1): a cleavable signal sequence of 19 aa, (2) a pro portion of 849 aa, including repeats I and II, (3) the mature enzyme containing both phlorizin hydrolase catalytic sites (repeat III) and lactase active site (repeat IV), (4) membrane-spanning hydrophobic segment serving as the membrane anchor, and (5) a cytosolic hydrophilic segment at the C-terminus (2, 27, 31, 32) (Figure 2a).

2.2 Biosynthesis

The cleavage of the 19 aa signal sequence of pre-pro-LPH to pro-LPH takes place in the endoplasmic reticulum (ER) and occurs during the process of translocation of the pro-LPH over the ER (33, 34) (Figure 2b I). The N-terminal sequences of pro-LPH are similar in human, rat and rabbit (2). The pro-LPH becomes *N*-glycosylated and forms homodimers in the ER (35, 36). The dimerization involves the C-terminal transmembrane domain and the cytoplasmic tail region (35). After that the pro-LPH is transported to the Golgi complex where it is complex- and *O*-glycosylated (37) (Figure 2b II). Glycosylation is needed both for enzymatic activity as well as for intracellular transport (37-40). The pro-sequence is cleaved off, in the *trans*-Golgi

complex or in a later compartment (41), in various steps by furin or a furin-like convertase before transport to the brush border membrane (34). This processing differs between the species studied, most likely due to the presence or absence of the furin and furin-like consensus sequences in the pro-LPH (2). The only function of the pro-sequence seems to be that it is needed for LPH to exit the ER, i.e. to pass the quality control of the secretory pathway (42-44). Lactase expressed in COS cells without its pro-sequence is retained in the ER (43, 44). The final proteolytic cleavage of LPH is carried out in the microvillus membrane by pancreatic trypsin (45) (Figure 2b III).

Figure 2. Structure and biosynthesis of LPH. (From ref. (36)).



2.3 Expression at organ and cellular levels

Expression of LPH is restricted to the small intestine of all mammals investigated (29, 36), making it a commonly used marker for differentiated enterocytes. In humans, the expression of lactase increases from the pylorus to the jejunum, with a maximum at 25% of gut length, it decreases from 50-70% of the gut length and after that remains stable (46). The mature LPH enzyme is positioned at the crypt/villus junction and on the villus of the enterocytes. It is anchored to the brush border membrane by its C-terminal transmembrane domain. Phlorizin hydrolase activity is more distal and lactase activity is closer to the membrane. The location of lactase at the tip of microvilli makes it most vulnerable to intestinal diseases which cause cell damage in comparison to the other disaccharidases, which are located deeper (14).

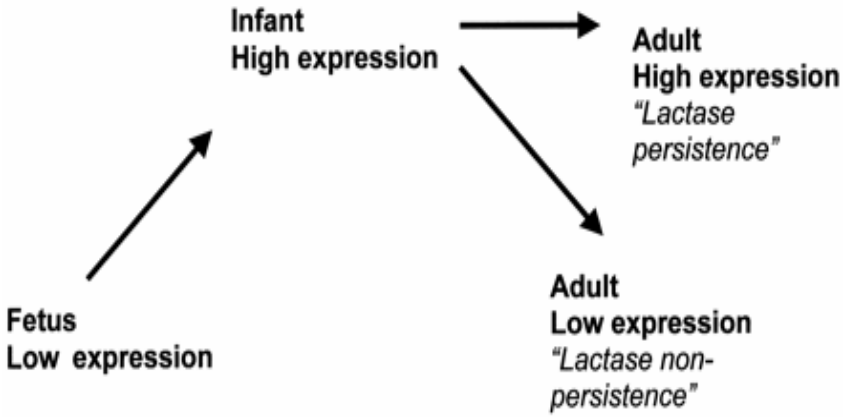
2.4 Expression during development

Lactase-phlorizin hydrolase is a critical enzyme for neonates that depend on their mother's milk for nourishment. In most species, including the majority of the human population, the LPH activity diminishes when mother's milk is not longer necessary for nutrition. In the human fetal small intestine LPH becomes expressed between gestational weeks 9 and 10 when the transition from undifferentiated endoderm to a columnar intestinal-type epithelium occurs (47). The expression of LPH is relatively low until approximately 24 weeks, with a gradual increase from then on until late gestation, after which a marked increase occurs around 32 weeks of gestation. The increased activity remains until early infancy, but decreases somewhat in the first year of life (48, 49). The obtained level of expression of LPH is then maintained during childhood (47).

Depending on the population, at some point during childhood or adolescence, LPH activity declines to approximately 10% of the activity of childhood levels (lactase non-persistence, adult-type hypolactasia). This is the case for the majority of the world's population, whereas in the rest, lactase activity persists throughout life (lactase persistence) (Figure 3). The age of the genetically determined decline of lactase activity has been difficult to study due to the difficulty in obtaining intestinal specimen from healthy children. However, in populations with high prevalences of

adult-type hypolactasia, the condition seems to manifest at an early age (50). In some Asian populations, where adult-type hypolactasia prevalence is as high as 100%, adult-type hypolactasia manifests as follows: in Thai children before two years (51), in Bangladesh before three years (52) and in Chinese children between three and eight years of age (53, 54). In African populations, lactase activity decreases in children beginning from three years of age (55) and in the majority (86%) of Somalian children lactase activity has been observed to be low (< 20 U/g protein) at ages > five years (56). In Peru (57) and Jamaica (58), 80% of the children were considered to be lactose maldigesters by the age of three years. In Israeli children manifestation was mostly between three and six years, but even at ages up to 12-16 years (59). In children of Caucasian origin, manifestation of lactose malabsorption (LM) seems to occur at a later age: in Greek children the prevalence of LM increases linearly between the ages of five and 12 years (60), and in the Finnish population between five and 20 years (61-63).

Figure 3. Lactase expression during development.



2.5 Regulation of LPH expression

The lactase (LCT) gene is regulated at several levels: 1) at the cellular level during differentiation of the enterocyte, 2) at the organ level for tissue specific and differential expression along the longitudinal axis of the small intestine, and 3) during development (36).

2.5.1 At the cellular level

Lactase expression is patchy in the distal parts of the small intestine in rat (64) and rabbit (65) after weaning. In humans with adult-type hypolactasia, this phenomenon is observed both at the enzyme and mRNA levels (65, 66). This implies a complete turn-off of lactase expression in the majority of enterocytes instead of a general downregulation of lactase expression in all enterocytes. Moreover, lactase mRNA is observed to be strictly localized in the cytoplasm below the apical membrane of the enterocyte, but the reason for the restricted localization is not understood (36, 67).

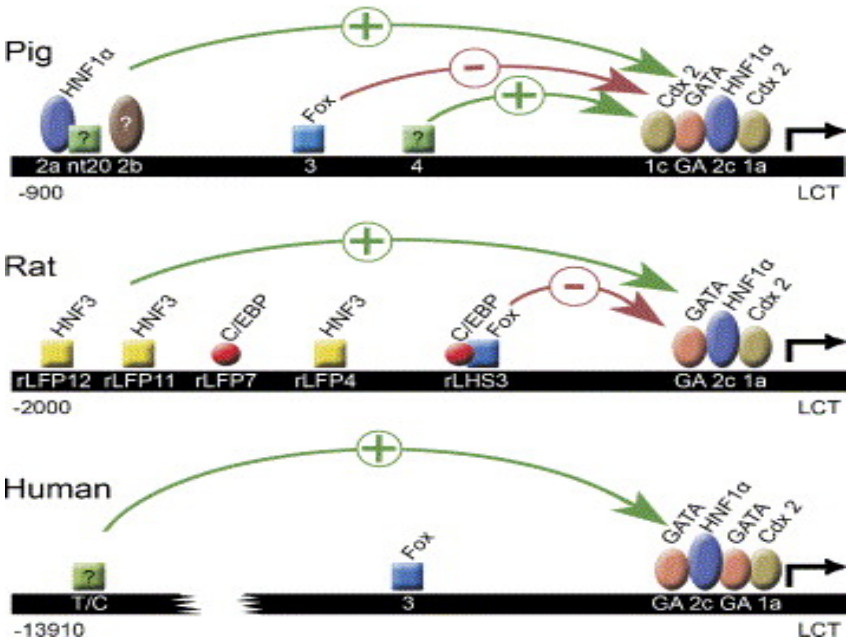
2.5.2 During development

The downregulation of lactase activity during childhood in humans is commonly considered similar to the post-weaning decline of lactase expression in mammals, e.g. the baboon (36, 68). The developmental decline in lactase activity seems mainly to be regulated at the transcriptional level in humans (69-74), in rat (75, 76), pig (77) and sheep (78). However, regulation at posttranscriptional level, too, has been suggested in humans (73, 79-82) and in rat (83). Some lactase non-persistent subjects have high expression of lactase mRNA (79, 81). In these rare subjects, the underlying factors may be posttranscriptional and posttranslational regulation causing mRNA degradation or failure of intracellular processing of the synthesized LPH enzyme, such as slow transport of the transcript, failure in maturation of the enzyme or failure to reach the membrane (81).

Sequence alignment of the lactase promoter in human, pig, rabbit, mouse and rat shows a conserved region of 150 bp just upstream of the transcription initiation site (36). Several regulatory transcription factor -binding sites (*cis*-elements) have been identified in the promoter region of pigs (84-86), rats (87) and humans (88, 89). Moreover, numerous transcriptional regulators in pigs (84, 86, 90), rats (83, 87) and humans (88, 89, 91-94), respectively, activating transcription from the LPH promoter, have been recognized. The pig 0.9 kb upstream sequence includes at least six *cis*-elements, of which two, CE-LPH1a and CE-LPH3, show 100% sequence similarity to human. Three other elements, CE-LPH1b, CE-LPH2c and CE-LPH4 are also well conserved between pig and human (86). An intestine-specific transcription

factor, Cdx-2, as well as another homeodomain protein, HOXC11 (91), interact with the LPH promoter through the TTTAC sequence of CE-LPH1a (94) and activate reporter gene transcription. HNF1α, a transcriptional activator, binds the CE-LPH2c element and when co-transfected with HOX11 produces 7-19-fold stimulation of transcription. A direct protein-protein interaction between Cdx2 and HNF1α leads to an increased level of gene activation (92). The regulator binding CE-LPH3, on the other hand, is a repressor that binds a member of the FREAC family (86). A GATA-binding site element has been identified in rat to locate between –73 and –100 bp; GATA-4 and GATA-6 interact with this element and activate transcription from lactase promoter in intestinal cells (89). GATA-5 and HNF1α also show co-operative activation of LPH expression (94). In rat, HNF3β binds at three sites enhancing LPH expression (87). Interestingly, the binding capacities of nuclear factors for the CE-LPH1 element and for HNF1α binding site in LPH promoter change during the post-weaning period (84, 86, 92, 95). In pig, a 100 bp region around position –850 upstream of the lactase gene bound with HNF-1α and an unidentified factor, has been observed to be necessary for high expression of lactase in Caco-2 cells (96). This sequence, however, has not been found in humans or rats. Unfortunately, comparisons of studies on the distal regulatory elements from other mammals to humans is rather complicated due to the fact that the 5'- flanking region of the human LPH gene contains five inserted stretches of repetitive DNA (97) (Figure 4).

Figure 4. Regulation of LPH expression during development. (From ref. (36))



3. LACTASE DEFICIENCIES

3.1 Terminology

Hypolactasia is term for a very low activity of lactase in the jejunal mucosa, whereas alactasia and lactase deficiency mean a total lack of lactase activity. Lactose malabsorption or lactose maldigestion imply a poor lactose digesting capacity. Congenital lactase deficiency (CLD) is the most severe form of lactase deficiency and manifests in neonates fed breast milk. Adult-type hypolactasia (lactase non-persistence) is a genetically determined condition. It is the ancient phenotype and characterized by decline in lactase activity during childhood. Adult-type hypolactasia causes primary lactose malabsorption (98). Secondary lactose malabsorption is caused by other reasons than genetically determined adult-type hypolactasia, such as microbial infections or celiac disease that damage the intestinal villi (99). Lactose intolerance refers to symptoms after lactose ingestion, and milk intolerance simply means gastrointestinal symptoms after milk ingestion (98) (Table 1).

Table 1. Terminology of lactase deficiencies.

Hypolactasia	Very low lactase activity
Alactasia/ lactase deficiency	Total lack of lactase activity
Lactose malabsorption/ lactose maldigestion	Poor lactose hydrolysing capacity
Congenital lactase deficiency	Rare disease of newborns
Primary lactose malabsorption/ lactase non-persistence/ adult-type hypolactasia	Manifests during normal development, irreversible
Secondary lactose malabsorption	Caused by mucosal injury, reversible
Lactose intolerance	symptoms from lactose
Milk intolerance	symptoms from milk

3.2 Congenital lactase deficiency (CLD)

Congenital lactase deficiency (CLD) is characterized by an almost total lack of lactase activity in the intestinal mucosa. CLD is a rare disorder and affects approximately 1:60 000 newborns in Finland (100). It is considered to belong to the so-called Finnish disease heritage, that have been enriched in the Finnish population due to the founder effect and genetic drift (101-103). Single cases of CLD have been reported outside Finland (104). Congenital lactase deficiency manifests as a watery diarrhea during the first days of life of an infant fed lactose-containing milk. Despite their good appetite and absence of vomiting, the diarrhea and loss of nutrients is so comprehensive that at the time of diagnosis children with CLD usually weigh less than their birth weight and suffer from dehydration and acidosis (100). The lactase activity in jejunal biopsies is observed to range from 0 to 10 U/g protein (100, 105). On a lactose-free diet the children, though, grow and develop normally (100).

3.2.1 Genetics of CLD

CLD is inherited as an autosomal recessive trait (100). A genealogical study revealed that CLD is enriched in eastern and northern Finland. The gene locus of CLD was assigned to chromosome 2q21 in Finnish families. The analyses of ancient haplotypes and linkage disequilibrium initially restricted the CLD region to a locus >2 Mb upstream of the LPH gene (106). However, detailed analysis of the haplotypes in the region and sequencing of the LPH gene resulted in identification of five mutations in the coding region of the LPH gene. A nonsense mutation c.4170T→A (Y1390X), Fin_{major}, predicting an early truncation of the lactase gene, was found in 84% of the patients. The four other mutations were rare. Two of the mutations resulted in a predicted frameshift and premature truncation at S1666fsX1722 and S218fsX224, respectively, and the two other in amino acid substitutions Q268H and G1363S (105). The carrier frequency of the Fin_{major} mutation was observed to be 1:35 in central Finland (105, 106).

3.3 Adult-type hypolactasia

Adult-type hypolactasia unlike CLD is not a disease but part of the normal development of mammals. At the time of weaning, the lactose content of food falls rapidly, and activity of lactase becomes unnecessary. In humans, lactase activity declines during childhood to approximately 10% of the activity at birth. Lactase non-persistence is indeed the more ancient phenotype in the human history, and those with high adult lactase activity carry the mutation (107). Subjects with lactase persistence are frequent in European populations and their descendents on other continents (108).

3.3.1 Clinical features

Hypolactasia, in most cases, leads to symptoms of lactose intolerance when a person with low lactase activity consumes lactose-containing food. The classic symptoms of lactose intolerance include abdominal bloating and pain, fullness, cramps, borborygmi, flatulence, loose stools and diarrhea (14, 109, 110). The former symptoms result from an increased motility of the intestine and an increased gas production due to bacterial fermentation of the unhydrolyzed lactose in the colon; a mechanism developed in order to conserve the nutritionally important calories. Loose stools and diarrhea, on the other hand, are due to an osmotic effect caused by unhydrolyzed lactose, leading to an increased secretion of water and electrolytes into the intestinal lumen until osmotic equilibrium is reached (109, 111). An increase in peristalsis and the hyperemic and edematous mucosa has been observed in jejunoscopy on subjects with adult-type hypolactasia following a lactose challenge (112).

The development of symptoms of lactose intolerance is individual. Interestingly, marked differences also exist at the population level. The severity of the symptoms has a correlation with the amount of lactose consumed, but also with the diet with which lactose is consumed, the rate of abdominal emptying, the small-intestinal transit time, individual sensitivity to the stretching of the intestinal wall as well as the degree of adaptation to lactose developed (14, 109, 110). Delayed gastric emptying has been observed to improve tolerance to lactose (113). Children are more prone to abdominal symptoms as well as loose, fluid stools because the passage of the lactose

content through small intestine and colon is normally more rapid. In adults diarrhea is often avoided, because the dietary load of lactose is smaller in relation to body weight, and there is more time for reabsorption within the colon (114). The composition of the colonic microbiota probably has a marked effect, although the factors affecting it are unknown. In hypolactasic subjects who tolerate lactose well large intestines densely colonized with anaerobic bacteria such as those of the *Bacteroides* group have been observed (115). β-galactosidase is the bacterial enzyme, which catalyzes the first step of lactose fermentation in the colon. β-galactosidase activity may vary 4-fold among the lactose-fermenting bacteria (1). A recent study tackled the role of colonic microbiota in lactose intolerance symptomatology by studying the amount and composition of the bacteria with β-galactosidase activity in subjects with primary low lactase activity and their controls. There was, however, no difference either in the percentage or the composition of the bacteria with β-galactosidase activity or β-galactosidase activity in feces between the tolerant and the intolerant groups (116).

It seems obvious that genetic factors have more or less effect on the clinical outcome of lactose intolerance. In some families with diagnosis of lactose malabsorption, intolerance does not seem to occur at all or the symptoms are very mild, whereas in other families an evident accumulation of the most symptomatic forms of lactose intolerance is observed (109, 117). Lactose intolerance may also present among some lactose absorbers after the ingestion of lactose, although the reason for this is unclear. It is probable that at least in some of these subjects some other underlying gastrointestinal disturbance, such as irritable bowel syndrome, is misattributed to lactose intolerance (14, 109, 114, 118).

3.3.2 Management

Lactase is a non-adaptable enzyme (119), thus the basis of the treatment of lactose intolerance is to reduce the amount of lactose in the diet; the degree of the restriction depends on the individual's tolerance (120). The majority of adults with lactose malabsorption tolerate 100 ml of milk, equaling about five grams of lactose without symptoms (121-123). Interestingly, lactose-free milk has been shown to induce symptoms in as many lactose malabsorbers as a milk containing seven grams of

lactose (123). Furthermore, chocolate with varying amounts (two-12 g) of lactose was equally well tolerated by subjects with self-reported lactose malabsorption (124), suggesting that small amounts of lactose do not play a significant role in the symptomatology of lactose intolerance. A cup of 200-250 ml of milk causes symptoms in 30-75% of the subjects, depending on the study population (122, 125-128). The consumption of 500 ml of milk or 50g of lactose, such as in a clinical tolerance test, causes symptoms in 80-100% of subjects with lactose malabsorption (122, 127, 129).

Lactose is better tolerated when it is consumed with some other food or when it is divided between several meals (130). Lactose content varies between different dairy products, and for example cheese which has a low lactose content is well tolerated, meaning that not all milk products need to be restricted in the diet. In some countries, such as Finland, low-lactose products in which lactose has been pre-hydrolyzed, as well as lactose-free milk in which lactose is removed from the milk with chromatographic separation, are available. Furthermore, the absorption of lactose can be facilitated by exogenous lactase preparations (121).

3.3.3 Nutritional consequences

Milk has a high content of protein and calcium. The main nutritional consequences of lactose intolerance seem to be due to decreased intake of calcium resulting from avoidance of dairy products. Both children (131) and adults (132-135) avoiding dairy products have been shown to have lower dietary intake of calcium and impaired bone health. Moreover, several studies have shown an increased incidence of adult-type hypolactasia, although diagnosed with methods of varying sensitivity and specificity, among subjects with osteoporosis or bone fractures (136-139).

Apart from the decreased absorption of lactose, the effects of lactose maldigestion on the absorption of other nutrients seem minimal. Several studies have assessed the absorption of calcium from milk in lactase persistent and deficient subjects, but the results have remained controversial (140-144). The infusion-aspiration technique with phenolsulphthalein (PSP), that is a nonabsorbable ileal recovery marker, has been used to quantify flow through the terminal ileum after a test meal of milk in subjects

with high lactase activity and those with adult-type hypolactasia (140). After whole milk, more protein, calcium, magnesium and phosphorus were recovered from the ileum in lactase deficient subjects. The authors, however, concluded that as the age and sex of the controls and subjects differed greatly, and as the status of vitamin D metabolism was not monitored, the direct comparison of the groups was not appropriate (140). Using the double-isotope technique, with a test meal of lactose and calcium in water, Cochet et al, concluded that the effect of lactose on calcium absorption is dependent on intestinal lactase activity (141). In their study calcium absorption was increased in subjects with normal lactase activity, when accompanied with lactose, whereas in subjects with adult-type hypolactasia lactose decreased calcium absorption (141). Zitterman et al., on the other hand, used the stable-strontium test, and observed that lactose does not have a beneficial effect on calcium bioavailability in lactose tolerant subjects (142). Tremaine et al., applying the double-isotope technique showed that malabsorption of lactose does not affect calcium absorption. In fact, the mean calcium absorption from both lactose-hydrolyzed and unhydrolyzed milk was significantly greater in subjects with adult-type hypolactasia in comparison to the lactase persistent subjects (143). This was, however, expected to be a consequence of a lower dietary calcium intake in the lactase malabsorbers, since decreased calcium intake is known to cause a compensatory increase in calcium absorption (143).

3.3.4 Diagnosis

Although response to withdrawal of lactose from the diet should be excellent in lactose intolerance, the diagnosis of adult-type hypolactasia based solely on symptoms is inaccurate (4, 145). Due to the considerable proportion of adult-type hypolactasia as a cause of unspecific abdominal complaints and its high prevalence worldwide, several laboratory methods have been developed for diagnostic purposes. The diagnostic methods are direct, as in measurement of the disaccharidase activities in intestinal biopsies or indirect, such as in the oral tolerance test or the breath hydrogen test (4).

3.3.4.1 Measurement of disaccharidase activities

Measurement of intestinal disaccharidase activities is the golden standard for diagnostics of adult-type hypolactasia, however, it is not suitable for routine diagnostics. It is to be noted that the disaccharidase values are affected by several factors, including age and ethnicity of the subjects as well as the biopsy site. If the intestinal mucosa is damaged all disaccharidase activities diminish leading to secondary disaccharidase deficiency, which, however, disappears when small-intestinal changes heal up (2).

There is an abrupt gradient of disachharidase activity in the proximal duodenum (146, 147), thus the standardization of the biopsy site to obtain reproducible duodenal biopsies is essential. The duodenal biopsies should be obtained from a relatively fixed site approximately 10-20 cm distal to the ligament of Treitz (4) in order to avoid biopsies from too proximal parts of the duodenum that show overall low activities of all disaccharidases. The biopsy technique as such has no effect on the enzyme activity (148).

In Finnish subjects, the values of disaccharidase activities regarded as normal range from 20-140 U/g protein for lactase, 40-250 U/g protein for sucrase and 150-700 U/g protein for maltase, with a lactase/sucrase ratio > 0.3 (Reference intervals of the Laboratory of Hospital for Children and Adolescents, University of Helsinki).

3.3.4.2 Lactose tolerance test (LTT)

The lactose tolerance test is based on the determination of the increase in blood glucose in blood samples taken at intervals of 15 to 30 min up to two hours after an oral load of 50 g of lactose. A rise in blood glucose > 1.7 mmol/l is indicative of normolactasia and that of < 1.1 mmol/l for hypolactasia. Symptoms after the test also need to be recorded (4), and symptoms combined with a marginal rise of blood glucose indicate hypolactasia.

It has been estimated using assay of disaccharidases as a reference method that the specificity of LTT is 77-96% and sensitivity 76-94% (4). LTT has been observed to

result in false positive results in approximately 30% of children tested (5). Also, the test is not reliable in diabetics since abnormal blood glucose levels might affect the result (149). Delayed gastric emptying has been observed to cause false positive results, too (5, 150).

3.3.4.3 The lactose tolerance test with ethanol (LTTE)

In the lactose tolerance test with ethanol (LTTE), blood galactose concentration is determined in one single blood sample taken 40 min after an oral lactose load. Ingestion of ethanol is used to inhibit the conversion reaction of galactose to glucose in the liver, which otherwise would occur rapidly after lactose ingestion. Blood galactose concentration of < 0.3 mmol/l at 40 min after lactose and ethanol ingestion indicates hypolactasia. The measurement of galactose instead of glucose makes the test more specific (4, 61, 151). The specificity of the test has been evaluated to range from 96% to 100% and sensitivity from 81% to 100% (4). Furthermore, the test is suitable for diabetics as well, and is less vulnerable to changes in gastric emptying rates (4). It is however not allowed to use the test on children.

3.3.4.4 Breath hydrogen determination (BHT)

The breath hydrogen test is based on the determination of exhaled hydrogen produced by the bacterial flora in the colon after an oral lactose load. The samples of hydrogen, taken at intervals of 15 to 60 min for two to six hours, are collected, and the change in hydrogen concentration in the expired air determined by gas chromatography. In hypolactasia hydrogen concentration increases > 0.3 ml/min over the baseline (4).

The specificity of BHT varies between 89-100% and sensitivity ranges from 69-100% (4). Prior exercise (152), use of acetylsalicylic acid (153) or antibiotics (154), and smoking (155) will increase the rise in hydrogen concentration. In addition, some subjects may be colonized with bacteria that are incapable of producing hydrogen (156), whereas in some cases colonic bacteria may consume hydrogen (154) and produce methane (CH_4) from it (157). The possible presence of methanogenic bacteria should be determined by determination of the methane in the samples (157).

Table 2. The specificity and sensitivity of the diagnostic methods of adult-type hypolactasia with assay of disaccharidases as the reference method. (Modified from ref. (4))

Test	Specificity	Sensitivity
LTT	77-96%	76-94%
LTTE	96-100%	81-100%
BHT	89-100%	69-100%
Genetic test (C/T_{-13910})*	100%	93%

*) In Finnish subjects >12 years of age

3.3.5 Role in unspecific abdominal complaints

Unspecific abdominal complaints cause concern for a considerable percentage of both children and adults and are frequent causes for visits to primary health care centers (158-162). Irritable bowel syndrome (IBS) is a common functional gastrointestinal disorder, with symptoms very reminiscence of those for adult-type hypolactasia, but not explained by structural or biochemical abnormalities (118).

Numerous studies have explored the role of adult-type hypolactasia as a cause of abdominal complaints. In Finnish and Estonian studies hypolactasia was observed to occur significantly more often among adults with unspecific abdominal complaints than in the general population (117, 163). In another Finnish study after testing with LTTE, the percentage of subjects with adult-type hypolactasia among the IBS -group, however, was the same as for the whole study group (118). Adult-type hypolactasia seems to associate more often with IBS, the greater the frequency of lactase non-persistence is in the population. This is illustrated in two Danish studies: among Danish patients with IBS, LM was diagnosed in 20% of the study subjects (164), whereas in immigrant workers with IBS, originally from areas with high prevalences of adult-type hypolactasia, the frequency of LM was as high as 85% (165). Although

the number of study subjects in the latter was low, it illustrates a common problem among immigrants in Northern European countries: when the traditional diet is changed to a lactose-containing diet of the new country, gastrointestinal symptoms appear. Not only children but also adults may simply be unaware of intolerance to lactose (127, 166), further complicated by the nature of the condition: the symptoms are non-specific, vary in frequency, and the discomfort occurs only some time after lactose consumption. On the other hand, in populations with general awareness of lactose intolerance such as Finland, self-reported, subjective lactose intolerance has been observed to strongly relate to IBS (118). Nevertheless, it should always be kept in mind, that coincident lactose intolerance may modify the clinical outcome of some other (gastrointestinal) disease (114).

The role of adult-type hypolactasia as a cause of recurrent abdominal pain (RAP) in children is controversial. Some studies have shown a substantial role for adult-type hypolactasia in the symptoms of children with RAP (167-171), whereas other studies have concluded that adult-type hypolactasia seems to be of little importance in RAP (172, 173). Based on these studies, it seems that in populations with high prevalences of lactase non-persistence, adult-type hypolactasia appears to be a major cause of RAP (110).

3.3.6 Lactose malabsorption in secondary disaccharidase deficiency

Activities of all disaccharidases diminish in the presence of mucosal injury: the more severe the damage, the greater the decrease in enzyme activity (55, 99, 148, 174-177). Lactase, compared to the other duodenal disaccharidases, is located most distal on the villus, and thus is the first and usually the one most severely affected. This is referred to as secondary lactase deficiency (2). The activity of lactase in secondary hypolactasia does not decline to such low levels as in primary hypolactasia. The recovery, however, takes a longer period than that of villous structures (109). In active coeliac disease, the recovery of the lactase activity during a gluten-free diet occurs slowly (2, 99, 178). In malnourished infants lactase and sucrase activities are decreased concomitantly with the degree of villus atrophy. In these malnourished children lactase mRNA was reduced to 32% and sucrase to 61% of normal (177). The decrease in the activity of lactase is particularly marked in cases of malnutrition

combined with protein deficiency (109). Other causes of secondary lactase deficiency are for example gastroenteritis (infectious diarrhea) (55), chronic diarrhea (179), HIV or rotavirus infection (180, 181), giardiasis (182), extensive gastrointestinal operations (108) as well as antibiotics such as neomycin (183), which may cause villous atrophy. Alcohol has also been shown to decrease disaccharidase activities (184). However, as mentioned earlier, secondary disaccharidase deficiencies are reversible, and a recovery of the mucosa correlates with an increase in disaccharidase activities (99).

4. GENETICS OF ADULT-TYPE HYPOLACTASIA

4.1 Evolution of lactase persistence

Dairying is estimated to have originated approximately 7 000 - 10 000 years ago (11, 185, 186). That is when man began to utilize milk after weaning, and lactase persistence, contributing to the added nutrition from dairying, became advantageous (108). A culture historical hypothesis states that populations that adopted a culture that relied on milk as a main nutritional source co-directed their own biological evolution by creating a selection pressure for lactose tolerance. This hypothesis is supported by findings that the frequency of lactase persistence phenotype is strongly correlated with the dairying history of the population (107). Moreover, in cows, the highest gene diversity in milk protein genes is seen in Europe where there is a high prevalence of lactase persistence, suggesting a gene-culture co-evolution between cattle and humans (187). Furthermore, epidemiological studies have shown that the development of dairying indeed preceded selection for lactase persistence (188).

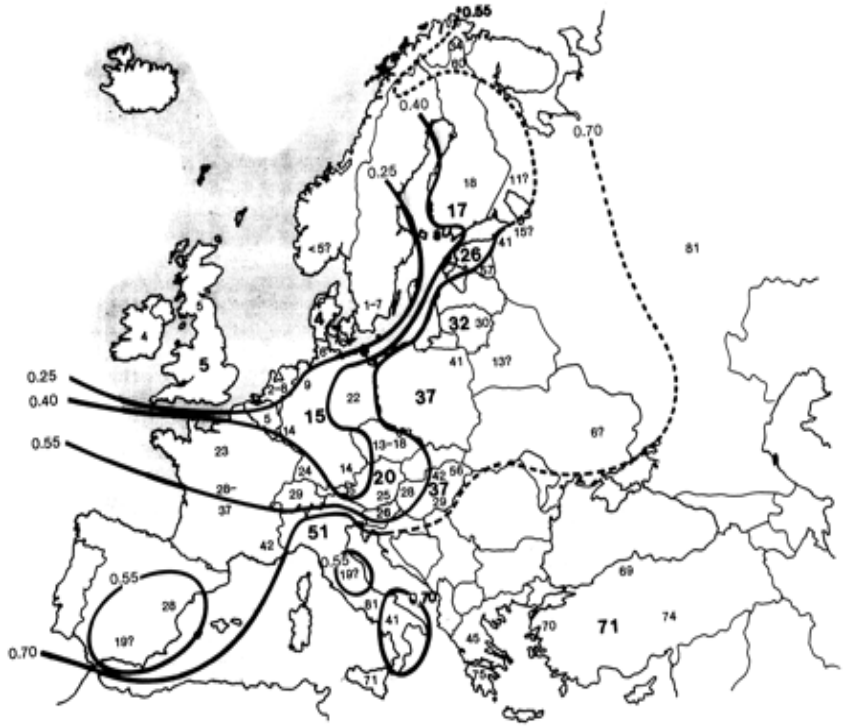
The haplotype carrying lactase persistence is estimated to having undergone strong positive selection during the previous 10 000 years (108, 186, 189-194) (Enattah NS, et al, unpublished data). This short time of only about 400 generations suggests a selective pressure that has been estimated to be among the strongest seen for any gene in the genome (191). This is supported by 1) the lactase persistence haplotype is exceptionally long (almost identical for nearly 1 Mb), and given it has a high frequency of 77% in Northern Europeans 2) the SNPs near the LCT gene show large

differences in allele frequencies among populations (191). Relative extended haplotype homozygosity (REHH) showed a very high genetic differentiation across this region between European Americans (dairying) and Asian/African Americans (non-dairying). This is suggestive for genetic hitchhiking of markers on the haplotype of lactase persistence (191). This study reported a selection-coefficient of 1.4 – 15% for lactase persistence, which is consistent with the 5% previously predicted using gene-culture co-evolutionary model (195).

4.2 Prevalence of adult-type hypolactasia

The prevalence of adult-type hypolactasia has marked variation between races and populations. The use of different indirect diagnostic methods of varying sensitivity and specificity, varying diagnostic criteria as well as in some studies considerably small numbers of participants, makes the evaluation of the studies on the prevalence of adult-type hypolactasia in different populations difficult (50, 128). The prevalence of adult-type hypolactasia is lowest in populations of Northern Europe. In Denmark and in Sweden the frequency of lactose malabsorbers is expected to be only around 1-5% (50). However, based on the molecular diagnosis (8) the prevalence in Sweden seems to be somewhat higher, around 10% (196). The regional prevalence rates in Finns are the best characterized of all the populations. The frequency of adult-type hypolactasia ranges from about 8% in Swedish-speaking Finns to 14% in Western Finland and finally to approximately 23% in Eastern Finland (8, 129, 197-199), with a mean of 18% in the capital region (Tikkakoski, submitted). The prevalence of adult-type hypolactasia is clearly higher in Southern Europe and is approximately 30% in Spain and 40-50% in Italy (50) (Figure 5).

Figure 5. Prevalence of adult-type hypolactasia in Europe. (From ref. (50))



In general, adult-type hypolactasia is more common in populations outside Europe. In Caucasians and their descendents in North America and Australia the prevalence of adult-type hypolactasia is low. In American whites, the prevalence is in the order of 15%, in African Americans about 80% and in Mexican American approximately 53% (50, 128). The prevalence in Latin America is generally high, around 70% in Mexico and 65% in Uruguay. In Asia, the prevalence is lower in the western parts: in Northern India around 30% and in Southern India 60-70%. The world's highest prevalences are in the populations of the Far East: in Thailand it is as high as 97-100%, and in Indonesia 91%. In China a prevalence of adult-type hypolactasia of approximately 90% is observed (50, 128).

The prevalence of adult-type hypolactasia in the black African populations ranges from 70 to 95%. Prevalence figures >90% are observed among populations with low milk consumption such as those in Nigeria and Zaire (50). However, interesting exceptions are the populations with tradition of milk consumption, nomads and the people who raise cattle, among whom the prevalence of hypolactasia is around 10-20% (50). For example, in nomadic Fulani in Nigeria the prevalence of adult-type

hypolactasia is 22% and in cattle-raising nomads in Beja, in northeastern Sudan, 17%. Among Nilotes, seminomadic cattle breeding tribes in southern Sudan, the prevalence is up to 75% (50, 200).

4.3 Adult-type hypolactasia- a genetically determined condition

Lactase non-persistence was in 1973 concluded to be controlled by an autosomal recessive single gene (7). These results were based on analysis of segregation of adult-type hypolactasia in Finnish families with the diagnosis of the lactase persistence status with LTTE, including subjects older than the manifestation age of adult-type hypolactasia in the Finnish population (7). Results of a Hungarian twin study supported the finding: the lactase phenotype had a complete concordance in monozygous twins. In dizygous twins the adult-type hypolactasia prevalence was compatible to the prevalence in the Hungarian population (201). A trimodal distribution in lactase activity, representing the homozygous recessive, heterozygous and homozygous dominant subjects was subsequently reported in several studies (202-204).

The observation of the trimodal distribution of lactase activity implied that the lactase persistence/non-persistence trait most likely was due to *cis*-acting differences, i.e. some polymorphism(s) within or near the lactase gene. This was supported by an expression study of the lactase mRNA transcripts from persistent and non-persistent individuals using polymorphisms residing in exons of the lactase gene as markers. The results clearly showed differential expression of the alleles of the lactase gene in subjects with intermediate lactase activities (71). Despite the cloning and the sequencing of the complete cDNA and 1 kb of the promoter region of the lactase gene, no sequence differences segregating with the lactase persistence trait had been identified until recently (27, 28, 74, 205, 206). Using the single nucleotide polymorphisms (SNPs) in the region four 60-kb haplotypes were identified, and three of them exist in Europe. One haplotype (haplotype A) associated significantly with high lactase expression (189). This haplotype was the most frequent in northern Europeans, consistent with the high frequency of lactase persistence in these populations (207).

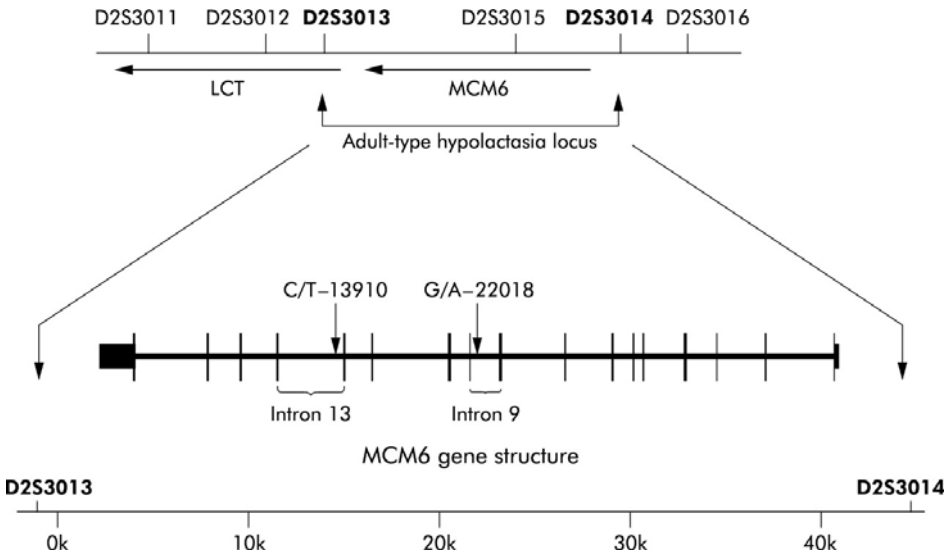
4.4 Identification of a DNA variant associated with adult-type hypolactasia

In an effort to identify the *cis*-acting variant associating with adult-type hypolactasia, Enattah et al. (8), to begin with, analysed the region flanking the LCT gene at 2q21 by genotyping seven polymorphic microsatellites in nine well-characterized Finnish families with known lactase persistence/non-persistence status (202). Traditional linkage analysis and using recombination events that define the centromeric and telomeric boundaries for the locus narrowed the region to approximately 5 cM. Nine polymorphic markers within this region that showed best evidence for linkage were chosen for fine mapping. Six markers spanning a 200 kb region showed highly significant evidence for linkage disequilibrium (LD): the strongest LD was detected on the LCT gene and 5' of the LCT gene, but no evidence for LD on markers 3' of the LCT gene. Based on analyses of constructed haplotypes within the families, using seven of the markers, the locus for lactase persistence was restricted to a 47-kb interval, covering only one gene, MCM6 (minichromosome maintenance 6). The restriction of the locus was made based on two chromosomes differing from the ancestral ones only at one marker. This restriction has been criticized (97, 190, 191) and claimed to have been caused by recent mutation at the marker instead of a recombination event. However, later on an identical prevalence of the specific allele at the marker in question among unrelated individuals (n=262) with lactase persistence as well as lactase non-persistence has been demonstrated (208).

Sequence analysis of the 47 kb region upstream of the LCT gene resulted in the identification of a total of 52 non-coding variants. Two of the variants, C to T_{-13910} (dbSNP rs4988235), and G to A_{-22018} (dbSNP rs182549), showed complete co-segregation with lactase persistence. The C/T variant is located 13,910 base pairs from the initiation codon of the LCT gene, in intron 13 of the MCM6 gene, and the G/A variant 22,018 base pairs upstream of LCT, in intron 9 of MCM6 (Figure 6). Further analysis of the variants in Finnish as well as in non-Finnish subjects with biochemically determined lactase activity, gave further support for the complete association of the C/T_{-13910} polymorphism with the lactase persistence/non-persistence trait. The G/A_{-22018} did not segregate with lactase activity in all cases suggesting that it was only in LD with the causing variant. Furthermore, the prevalence of the C/C_{-13910} genotype in samples from Finnish, French, North American and African

Americans subjects studied was consistent with the epidemiological data on the prevalence of the adult-type hypolactasia in the population in question. Accordingly, the C/C$_{-13910}$ genotype, based on these findings, was concluded to associate with adult-type hypolactasia, whereas subjects with the genotypes C/T$_{-13910}$ and T/T$_{-13910}$ were shown to be associated with lactase persistence (8).

Figure 6. Location of the SNPs suggested being associated with adult-type hypolactasia. (From ref. (8)).



4.5 Functional studies

Since the identification of the C/T$_{-13910}$ variant associated with adult-type hypolactasia several studies have explored its functional significance. Quantitation of relative expression of the LPH mRNA transcripts from the C$_{-13910}$ and T$_{-13910}$ alleles by allele-specific reverse transcription polymerase reaction (RT-PCR) in Finnish adults, showed several times higher expression of LPH mRNA from the T$_{-13910}$ allele (209). Two groups have separately studied the gene regulatory activity of the -13910 region by transfecting the intestinal Caco-2 cell line with constructs of the -13910 region, lactase promoter and luciferase reporter gene. Both studies showed that the –13910 region contains a strong enhancer, the T$_{-13910}$ variant enhancing the LPH promoter more strongly (96, 210). Furthermore, in electrophoretic mobility shift assay (EMSA) nuclear protein interaction showed a stronger nuclear factor binding to the T$_{-13910}$

compared to the C_{-13910} allele (96, 210). Recently, transcription factor Oct-1 and glyceraldehyde-3-phosphate (GADPH) were co-purified by DNA affinity purification using the sequence of the T_{-13910} variant (211). In supershift analysis, Oct-1 bound directly to the T_{-13910} allele. Co-expression with HNF1α stimulated lactase gene expression. GADPH was suggested to interact with Oct-1. In addition, binding sites to intestinal transcription factors GATA-6, HNF4 α, Fox and Cdx-2 were also identified in the –13910 region, providing further support that this region underlies the developmental regulation of lactase expression in human (211).

The function of the MCM6 (minichromosome maintenance 6) gene where the C/T_{-13910} variant is located is relatively poorly known. MCM6 is the human homologue of a yeast cell division cycle gene, showing similarity to rat intestinal crypt-cell licensing factor. MCM6 transcripts are expressed in all tissues studied, both in fetuses, children and adults. Accordingly, MCM6 is not restricted in its tissue distribution and does not show age-related variation in the level of expression in the human intestine, in contrast to the expression of lactase gene (212).

4.6 Correlation of the C/T_{-13910} genotypes with the phenotype

During the time the work was being done for this thesis, a few studies have assessed the correlation of the C/T_{-13910} genotypes with the lactase persistence phenotype in populations outside Finland. A common weakness in these studies has, however, been the lack of the biochemically determined lactase activity as a definition of the lactase persistence/non-persistence status of the subjects studied. As previously discussed, the results of the indirect tests are not reliable in all cases.

In a study with Austrian subjects, the 24% frequency of the C/C_{-13910} was concordant with the frequency of lactose intolerance diagnosed by the breath hydrogen test (BHT) in individuals from the same region (137). In another Austrian study, a 97% correlation was observed between the C/C_{-13910} genotype and a positive test result in BHT. Of those with C/T_{-13910} and T/T_{-13910} genotypes 14%, however, had a positive BHT (213). In a German cohort, the frequency of the C/C_{-13910} genotype was 21.4%; somewhat higher than that diagnosed by BHT (15%) (214). In a Swedish study, the results from LTT correlated perfectly with the genotyping results in subjects C/C_{-13910}

and T/T$_{-13910}$ genotypes, whereas in three subjects with C/T$_{-13910}$ genotype the result in LTT was suggestive of lactose malabsorption (215). Another Swedish study gave similar result: in 94% of the cases the results on genotyping and LTT were concordant (216). The results from a British laboratory showed perfect association between the C/T$_{-13910}$ genotypes with lactase persistence/non-persistence phenotypes in Northern European samples studied but not in 2/40 Southern European samples (190, 217). Complete correlation of transcript expression level and the C/T$_{-13910}$ genotype, could neither be demonstrated (190, 217).

A recent study reports the C/T$_{-13910}$ variant frequencies in 20 distinct African cultural groups (218). For seven of the 20 groups phenotype data was available in the literature. In the sub-Saharan groups the T$_{-13910}$ allele was found too seldom in order to underlie the lactase persistence phenotype, thus it was suggested that C/T$_{-13910}$ might not be the causing variant in these groups. (218). Despite the fact that the definition of the phenotype was not reliable in this study it seems possible that another variant may underlie the lactase persistence in those populations; however, more studies on the issue are warranted. Taken together, these data suggest an excellent correlation between C/T$_{-13910}$ polymorphism and lactase persistence/non-persistence phenotype in all populations- except for the sub-Saharan Africans- studied so far.

5. DISEASES ASSOCIATED WITH DAIRY PRODUCT CONSUMPTION

5.1 Cow's milk allergy (CMA)

Cow's milk allergy (CMA) is an immunologically mediated adverse reaction to cow's milk proteins, which usually develops soon after introduction of cow's milk into the diet. CMA affects approximately 2-3% of infants during the first two years of life (219, 220). In most cases CMA is transient: approximately half of the cases have developed tolerance to cow's milk by three to four years of age (221). In a Finnish follow-up study 15% of the subjects at a mean age of 8.6 years had not recovered from CMA (222). Adult-onset CMA is rare (<1%) and usually much milder than in infants (223). The treatment of CMA consists basically of as strict as possible avoidance of cow's milk (223).

The casein fraction (αS_1-, αS_2-, β-, and κ-casein) accounts for 80% of cow's milk proteins. The whey fraction (20%) contains globular proteins of which the major ones are α-lactalbumin and β-lactoglobulin. Bovine serum albumin (BSA) and lactoferrin are detected in smaller amounts. In up to 75% of the subjects with CMA, polysensitization occurs to several proteins, thus no single protein or protein structure can be taken to account for the major part of milk's allergenicity (224). Possible contamination of lactose with trace amounts of milk proteins is the reason why infant formulas used in the treatment of CMA do not contain lactose. However, children with CMA are clinically tolerant to lactose and need not to exclude foods or pharmaceutical preparations containing lactose (225).

5.1.1 Clinical features of CMA

The symptoms of CMA involve several organs: skin (urticaria), respiratory tract (rhinitis) and gastrointestinal tract (most commonly vomiting, pain, diarrhea), but in the worst cases CMA may result in anaphylactic shock (226). The time when the symptoms appear varies from minutes to hours; delayed-type reactions take days, or even up to two weeks. The reactions of the skin are usually immediate, whereas in the delayed-type reactions gastrointestinal symptoms predominate (227). The immediate-type reactions are usually IgE-mediated, whereas the mechanisms which associate with the delayed-type reactions are poorly understood, and only some of the reactions are IgE-mediated (219). Based on a recent follow-up study, all children diagnosed with a non-IgE CMA had become tolerant to cow's milk by the age of five years, but in those with IgE-mediated CMA, the hypersensitivity often persisted up to school age (222). Cow's milk protein-sensitive enteropathy (CMSE), on the other hand, is a condition, which manifests mainly in the gastrointestinal tract with the typical findings in endoscopy being visible lymphonodular hyperplasia of the duodenal bulb and lymphoid follicles without villous atrophy (228). CMSE may persist or manifest for the first time in older children. The prevalence and significance of this condition, however, is this far poorly understood (228).

Table 3. Features of adult-type hypolactasia and cow's milk allergy. (Modified from ref. (223))

	Adult-type hypolactasia	**Cow's milk allergy**
Prevalence	High	Low
Racial variation	High	Low
Age	Childhood/Adulthood	Infancy
Offender	Mammalian milk sugar	Bovine milk proteins
Mechanism	Enzyme deficiency	Immunologic
Symptoms	GI	GI, skin, respiratory

5.2 Colorectal carcinoma (CRC)

Colorectal carcinoma (CRC) is one of the most frequent types of cancer in Western industrialized countries. In comparison with other countries, Finland has low colon cancer rates in both genders (229). CRC is causally related to both genetic susceptibility and environmental factors. To mention a few, p53, DNA mismatch repair (MMR) genes, H-ras, and the genes of the APC- β-catenin- Tcf –pathway are often mutated in CRC (230). In the classic pathway of the adenoma-carcinoma sequence accumulation of several mutations –including those activating oncogenes as well as those inactivating tumor-suppressor genes– is considered a requisite for carcinoma development (231, 232). It is commonly agreed that the risk of colorectal cancer is modified by food and nutrition. Among the environmental factors meat and alcohol consumption and smoking are regarded as significant risk factors, whereas inverse association has been observed with the consumption of vegetables, physical activity and non-steroidal anti-inflammatory drugs (NSAIDs), among others (230). There are numerous theories on the interactions of the specific molecular and environmental factors behind CRC development (230).

5.2.1 Dairy products in CRC

Various epidemiological studies have tackled the role of dairy products in the development of CRC, and several cohort studies suggest a modestly reduced risk of CRC with the consumption of dairy products (229, 233, 234). It is of interest that two of them suggesting a protective effect (229, 233) were carried out on the Finnish population, in which the milk consumption is among the highest in the world (235). In theory, several components of dairy products could increase or decrease the risk of CRC. The following different components of the milk have been suggested to mediate the protective effect: calcium (229, 234, 236-240), vitamin D (234, 236-240), lactose (233), lactoferrin (241) and conjugated linoleic acid (242). Milk fats, particularly the saturated fats, may increase the CRC risk (234). The role of the individual components on the carcinogenic process, however, is extremely difficult to dissect.

5.2.2 Role of colonic microbiota

Colonic microbiota, whose main function is to degrade and ferment potential energy sources reaching the colon, is a major environmental factor that modulates the risk of colorectal cancer (243). Some of short-chain fatty acids produced in degradation have been proposed to be related to colorectal carcinogenesis (244). Probiotics are cultures of live microorganisms which, when applied to animal or man, beneficially affect the host by improving the properties of the indigenous microbiota. In animal models treatment with probiotics has been shown to reduce the prevalence of CRC (245). Prebiotics, in contrast, are non-digestible food components, that reach the colon in an intact form, and stimulate the growth or activity of bacteria such as *Lactobacilli* and *Bifidobacteria* (246). Some of these strains bind the surface of colonic epithelial cells in culture, and thus may hypothetically protect the surface by facilitating repair and reducing irritation at the colonic epithelium (234). In low risk populations of CRC high concentrations of some of *Lactobacilli* species and *Eubacterium aerofaciens* have been observed in comparison to high risk populations (247, 248). In subjects with lactose malabsorption, lactose has been suggested as a natural prebiotic, and therefore might be of relevance in the pathogenesis of CRC (246). Lactose malabsorbers consuming dairy products have thus been suggested to have less of a risk for CRC (249).

AIMS OF THE STUDY

The aims of this study were:

1) To study the timing and mechanism of developmental downregulation of lactase activity during childhood
2) To assess the applicability of the genetic test of the C/T_{-13910} variant as a screening method for adult-type hypolactasia
3) To clarify the prevalence of various gastrointestinal symptoms and consumption of milk products in Finnish children and adults with molecularly defined adult-type hypolactasia
4) To study the relationship between adult-type hypolactasia and the development of colorectal carcinoma

MATERIALS AND METHODS

1. STUDY SUBJECTS

1.1 Age of lactase downregulation

This study included 329 children undergoing gastrointestinal endoscopy for abdominal complaints. Of them 252 were Finnish (mean age 9 years; range 0.6-20.2), 65 of Somalian origin (mean age 6.9 years; range 0.1-15.6 years) and 12 of other Caucasian origin (mean age 6.9 years; range 1.9-10.9 years). They all lived in Finland. Duodenal biopsies, 10-20 cm distal to the ligament of Treitz, were obtained in endoscopies at the Hospital for Children and Adolescents, University of Helsinki between 2001 and 2003. The biopsy sample was divided into two parts: one was used for routine histological examination and the second for assaying the disaccharidase activities and subsequent DNA extraction. Of the Finnish families 28% (n=71) completed a questionnaire about the child's milk consumption and possible milk related symptoms.

1.2 Mechanism of lactase downregulation

For this study, duodenal biopsy specimens from 45 Finnish children aged between 10 months to 23 years were obtained at the Hospital for Children and Adolescents, University of Helsinki in 2003-2004. The biopsy specimen was divided into three pieces: one for routine histological examination, the second for assaying disaccharidase activities and subsequent DNA extraction, and the third part for total RNA extraction. The specimens were analysed for the coding SNP G/A_{+593} on exon 1 of lactase (LPH) gene and those heterozygous for the G/A_{+593} (n=15) (age range: 10 months to 23 years, mean age: 7.4 years) were chosen for the actual study (Figure 7). The clinical diagnoses of these children were suspected gastroesophageal reflux (n=12), abdominal pain of unknown origin (n=1), *H.pylori* infection (n=1) and vomiting (n=1).

Figure 7. Flowchart of sample processing

Duodenal biopsies (n=45)

↓

Samples excluded because of low overall disaccharidase activities (n=8)

↓

Genotyping for SNPs C/T_{-13910} and the coding A/G_{+593}

↓

Genotype C/T_{-13910}	n (%)	Of which A/G_{+593} heterozygotes (n)
C/C	5 (13.5%)	3
C/T	19 (51.4%)	12
T/T	13 (35.1%)	0
Total	37 (100%)	15

↓

RNA extraction (A/G_{+593} heterozygotes)

1.3 Adult-type hypolactasia and cow's milk allergy

This study included 172 children (mean age 8.6 years) of whom 93 (54%) had a previous diagnosis of cow's milk allergy (CMA) and 79 (46%) were controls. They all had participated in a study, in which 6209 unselected infants born between 1994 and 1995 in the Helsinki region were followed from birth for the development of CMA (220). At the age of 8-9 years, during August 2003 to March 2004, these children were clinically examined at the Helsinki University Central Hospital and taken a blood sample for analysis of the C/T_{-13910} variant of adult-type hypolactasia. At that time families were also asked about the child's milk consumption and possible milk related symptoms.

1.4 Adult-type hypolactasia among Finnish adults

This study included 1900 working age adults (age range 18-64 years, mean age 46.5 years) from the capital area of Finland attending primary health care. They gave a blood sample from which DNA was extracted and used for genotyping the C/T$_{-13910}$ variant of adult-type hypolactasia. The participants also filled in questionnaires developed for this study. The questions concerned various gastrointestinal symptoms, the daily consumption of dairy products and possible milk related symptoms. The response rate was extremely high, 99.3%. The collection of the questionnaires and blood samples occurred during a three-month period through February to May 2004.

1.5 Adult-type hypolactasia and colorectal carcinoma

In this study DNA samples from a total of 2766 subjects were analysed for the C/T$_{-13910}$ variant of adult-type hypolactasia. The samples comprised 963 Finnish, 283 British and 163 Spanish subjects with colorectal cancer, and 773 Finnish, 363 British, and 221 Spanish control subjects. The Finnish controls were frequency matched to the cases, and derived from the same geographical provinces as the cases. The Spanish cases and controls were all derived from Catalonia, and the UK subjects were all primarily derived from Southern England. Clinical data, including age at the time of diagnosis, sex, tumour histology, stage and site of the tumour were obtained from 92% of the Finnish participants with colorectal cancer.

2. METHODS

Table 4. Methods used in this study.

Method	**Study**				
	I	**II**	**III**	**IV**	**V**
Assay of disaccharidases	x	x			
DNA extraction	x	x	x	x	
RNA extraction		x			
PCR/Minisequencing	x	x	x	x	x
Sequencing	x	x			
RT-PCR and quantitation mRNA levels		x			
Analysis of dairy consumption and GI-symptoms based on a questionnaire	x		x	x	
Statistical methods	x	x	x	x	x

2.1 Assay of intestinal disaccharidases

Lactase, maltase and sucrase activities were determined at the laboratory of the Hospital for Children and Adolescents, University of Helsinki, according to the method of Dahlqvist with modifications (6, 250). The assay of intestinal disaccharidases is based on the composition of disaccharides partly of glucose molecules, which are liberated on hydrolysis. The activities of the intestinal disaccharidases are assayed by incubating the intestinal samples with the respective disaccharides, after which the amount of glucose liberated is measured using Tris-glucose oxidase reagent and a direct correlation to the activity of the intestinal disaccharidase studied is obtained. Tris-buffer is used to inhibit contaminant disaccharidases (6, 250).

Accordingly, the duodenal biopsies were weighed, solubilized in cold NaCl (100 µl/mg tissue) and homogenized on ice for 45 seconds. After centrifugation (4500 rpm/15 min) supernatants were diluted in cold NaCl, pipetted on a Deep Well plate (ABgene, Epsom, UK), mixed by vortexing and incubated in 37˚C for 60 minutes. After that 300 µl of icecold glucose oxidase reagent (Sigma-Aldrich, Helsinki, Finland) was added to each well and incubated again in 37˚C for 60 minutes. Finally,

the absorbance of the samples was measured at 450 nm on a Multiscan spectrophotometer (Bio-Rad Laboratories, Espoo, Finland) and the activity of the disaccharidases calculated as units of enzyme activity. One unit (U) is defined as the activity of a disaccharidase required for hydrolyzing 1 μmol of disaccharide per minute. Lactase deficiency was diagnosed when the activity of lactase was < 10 U/g protein and lactase/sucrase ratio < 0.2 (4, 6, 163). The activities of maltase and sucrase were determined in order to exclude patients with secondary lactase deficiency.

2.2 DNA extraction

DNA extraction was carried out using standard protocols: by phenol-chloroform extraction for DNA extraction from duodenal specimens, and using Puregene kit (Gentra Systems, Minneapolis, USA) for DNA extraction from whole blood samples.

2.2.1 DNA extraction from duodenal biopsies

After the assessment of disaccharidase activities, the intestinal specimens were used to extract the DNA. The sample was moved into a proteinase-K buffer (0.5% SDS, 0.1M NaCl, 50mM Tris pH 8.1, 20mM EDTA) and proteinase K (20mg/ml) was added in order to dissolve the proteins. After an overnight incubation at 37˚C the DNA was extracted by phenol-chloroform extraction (251). Precipitation of DNA was done at –70 ˚C for 15 min after addition of 2.5 vol cold ethanol and 1/10 vol 3M NaAc. Finally the samples were centrifuged, washed with 70% ethanol, lyophilized and solubilized in dH_2O.

2.3 RNA extraction from duodenal biopsies

Total RNA was extracted using the standard protocol for the RNeasy mini kit (Qiagen, Crawley, West Sussex, UK) followed by DNase treatment.

2.4 Polymerase chain reaction (PCR) and reverse transcriptase PCR (RT-PCR)

DNA fragments were amplified by polymerase chain reaction –amplification. The following PCR protocol was used for amplification of the C/T_{-13910} variant followed by minisequencing: the PCR amplification was carried out in a 50 µl volume with 30-100 ng DNA, primers (one biotin-labeled (5 µM) and one unmodified (50 µM)), dNTPs (1000 µM), 0.5 U of Taq polymerase (Dynazyme, Finnzymes) in a standard buffer. The PCR cycle conditions used were as following: an initial round of denaturation at 94 ˚C for 4 min, then 35 cycles at 94 ˚C for 30 s, 53 ˚C for 30 s, 72 ˚C for 1.15 min, and a final extension of 72 ˚C for 10 min. The resulting PCR products were analyzed by 1.5% agarose gel electrophoresis to verify the amplification and the size of the PCR product. See table 5 for the primer sequences.

Table 5. Sequences of primers used for genotyping the C/T_{-13910} variant of adult-type hypolactasia.

Primer	Sequence (5'-3')
SNP C/T_{-13910} Forward primer	5'-(Biotin)CCTCGTTAATACCCACTGACCTA-3'
SNP C/T_{-13910} Reverse primer	5'-GTCACTTTGATATGATGAGAGCA-3'
SNP C/T_{-13910} Detection primer	5'-GGCAATACAGATAAGATAATGTAG-3'

In Study II, complementary DNA (cDNA) for lactase gene was reverse transcribed from total RNAs extracted from intestinal samples using 5′-TGTCGAATCTGCTCTAAGGAG primer. The 20 µl reaction mixture contained 10 mmol/l $MgCl_2$, 50 mmol/l Tris-Hcl (pH 8.3), 100 mmol/l KCl, 4 mmol DTT, 1 mmol dNTPs, 31 U RNase inhibitor (RNAguard, Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK), 20 pmol of the primer and 200 U of AMV (avian myeloblastosis virus) reverse transcriptase (Finnzymes, Espoo, Finland). RT reactions were performed at 50 ºC for 50 minutes. The PCR-amplifications of the cDNAs were performed using a biotinylated forward primer (5'-(biotin)ACCTAGTTGGGATCTGGTTCA) and an unmodified reverse primer (5'-AGATATGGGTGGTTCTAGCAG), and for a G/A_{+593} heterozygous (in exon 1 of

lactase gene) genomic DNA sample using a biotinylated forward primer (5'-(biotin)ACCTAGTTGGGATCTGGTTCA) and an unmodified reverse primer (5'-TGTCGAATCTGCTCTAAGGAG) with a PCR-protocol of 35 cycles at 55 ºC with a 30 second extension step at 72 ºC. The G/A_{+593} SNP in exon 1 of the lactase (LCT) gene was chosen because it previously had been shown to be the most polymorphic SNP of the LCT gene in the Finnish population (209).

2.5 Solid-phase minisequencing

In solid-phase minisequencing, firstly, PCR amplification with one biotin-labeled and one unmodified primer is performed. Two aliquots of the amplified sample are then attached to solid support coated with streptavidin. Denaturation is carried out with alkaline treatment, followed by a washing step where the excess dNTPs from PCR and the unbiotinylated strand are removed. After that a detection primer is hybridized to the single-stranded template adjacent to the variant nucleotide. DNA polymerase will extend the primer with the [^{3}H]-labeled dNTP if it is complementary to the nucleotide present at the variable site. In other words, in samples from homozygous individuals a labeled dNTP is incorporated in only one of the reactions and in samples from heterozygous individuals in both reactions. After the washing steps the sample is again denaturated with alkaline treatment, and the eluted radioactivity expressing the amount of incorporated label is measured using a scintillation counter. The results of the assay are numeric counts per minute (cpm), which express the amount of [^{3}H]dNTP incorporated in the minisequencing reactions. The ratio (R) between the cpm values directly reflects the ratio between the two sequences in the original sample (252, 253).

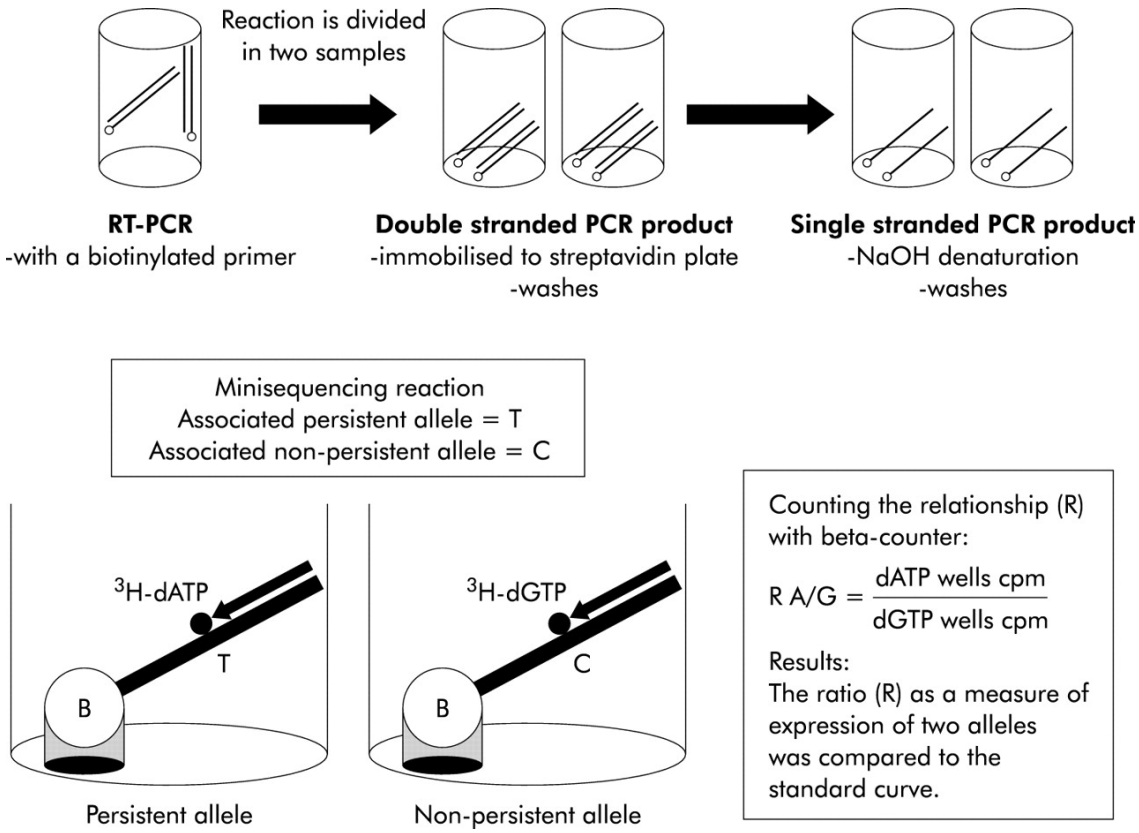
Briefly, in the minisequencing of the C/T_{-13910} single nucleotide polymorphism, two 10-µl aliquots of the biotin-labeled PCR product were captured to straptavidin coated microtitre wells (Thermo Electron, Helsinki, Finland). The reaction mixture contained 10 pmol of the detection primer (for sequence, see Table 5), 0.1 µl of either ^{3}H-dCTP or ^{3}H-dTTP (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and 0.05 U of DNA polymerase (Dynazyme II, Finnzymes, Espoo, Finland). The reactions were allowed to occur for 15 min at 56˚C before washing off the unattached label.

Finally, the attached detection primer was eluted by NaOH treatment and the radioactivity measured in a liquid scintillation counter (Rackbeta 1209; Wallac, Turku, Finland).

2.6 Quantitation of mRNA-levels by solid-phase minisequencing

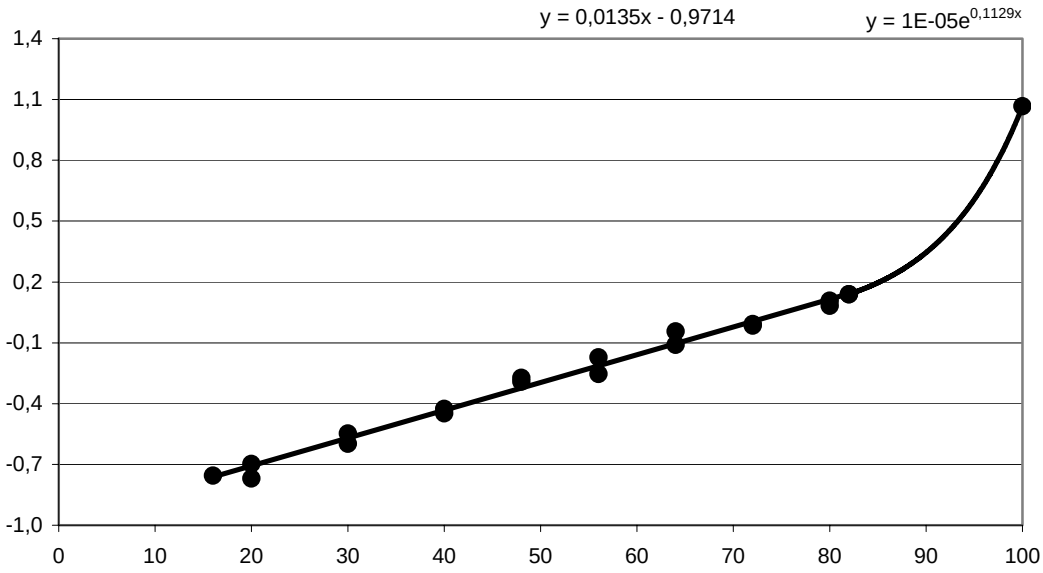
For preparation of the standard curve in Study II, two total RNA samples homozygous either for the G_{+593} or for A_{+593} allele were mixed at varying ratios (0%, 6%, 10%, 16%, 20%, 30%, 40%, 48%, 56%, 64%, 72%, 80%, 82% and 100% of AA_{+593}). Using protocols described above, the RNA mixtures were reverse transcribed to cDNA and amplified by PCR. The relative amounts of G and A alleles in these mixtures, in a genomic DNA sample in which the alleles are present in exact 1:1 ratio and in the actual study samples (n=15) (two reactions per sample) were assessed simultaneously by quantitative minisequencing. Each PCR-amplified cDNA product was divided into two 10 µl reactions and captured on streptavidin-coated wells with 40 µl of 20 mmol/l Na_2SO_4 (pH 7.5), 0.15 mol/l NaCl, and 0.1% Tween 20. The microtiter plates were incubated for 1.5 h at 37 °C after which the wells were washed, and then incubated at room temperature with 50 mM NaOH to denaturate the DNA. After removing the unbound strand by washing the reactions were carried out for 15 min at 51 ºC with 20 pmol of the detection primer (5'- TTTTCTGTGGGCATCACTGAG), 0.5 U of Taq polymerase in 1x PCR buffer and 2 pmol of tritium labeled dNTP (^{3}H-dGTP and ^{3}H-dATP). The wells were washed and treated with 50 mmol NaOH for 5 min. The incorporated tritium-labeled nucleotides were measured in a liquid scintillation counter (Rackbeta 1209; Wallac, Turku, Finland) (Figure 8).

Figure 8. Minisequencing in quantitation of mRNA levels. (From ref. (209))



Based on information of the relative amounts of G and A in the G/A_{+593} heterozygous genomic DNA sample, the concentrations of G and A in the RNA mixtures for the standard curve were calibrated, and an 11-point standard curve obtained. The standard curve was observed to be linear up to 82% ($y = 0.0135x - 0.9714$) and behaved exponentially for 82-100% ($y = 1x10^{-5}e^{0.1129x}$). The standard curve allowed us to relate the results of the minisequencing with the actual relative amounts of LCT mRNA from the two alleles, the C_{-13910} and the T_{-13910} present in the biopsy sample (Figure 9).

Figure 9. Standard curve. The amount of A_{+593} allele in total RNA plotted as a function of the GA_{+593} ratio on a log scale.



2.7 Sequencing

The DNA samples were amplified by PCR with the procedure as detailed above, sequenced using BigDye terminator chemistry, and run on an ABI377 automatic DNA sequencer according to the manufacturer's instructions (Applied Biosystems, Perkin Elmer, Foster City, California, USA). The analysis of the obtained DNA sequences was carried out using Sequencher 4.1.4 software (Gene Codes, Ann Arbor, Michigan, USA).

2.8 Assessment of gastrointestinal (GI) symptoms and dairy consumption by questionnaires

The participants in Studies I and III (either the children themselves or their parents) filled in questionnaires concerning their daily consumption of milk as a drink (normal and low-lactose milk), consumption of other dairy products, and possible milk related GI symptoms. In Study IV, we used a more comprehensive questionnaire in which the frequency of various GI symptoms and their relation to meals, as well as the

correlation of the symptoms to different type of milk products and other food was assessed. Questions on previous diagnosis of lactose intolerance or other GI disease such as colon cancer, celiac disease, or *Helicobacter pylori* infection were also included. The formulated questionnaire was pre-tested on a small group of healthy adults.

2.9 Statistical methods

All statistical analyses were performed using SPSS for Windows, release 11.5.1 (SPSS Inc., Chicago, Illinois, USA). Frequency differences were analysed with Pearson's χ^2 test or Fisher's exact 2-sided test.

RESULTS AND DISCUSSION

1. TIMING AND MECHANISM OF DOWNREGULATION OF LACTASE ACTIVITY DURING DEVELOPMENT

We applied the recently identified C/T$_{-13910}$ variant associating with adult-type hypolactasia as a molecular marker to study the timing and mechanism of developmental downregulation of lactase activity during childhood. The aim was, in the first place, to obtain information on whether the C/T$_{-13910}$ genotypes correlated with the biochemically determined lactase activities of children at various ages and ethnicities. Accordingly, if the C$_{-13910}$ allele indeed has a causative role in the development of adult-type hypolactasia, we should be able to detect the age at which lactase activity is downregulated in those children with the homozygous C/C$_{-13910}$ genotype. Furthermore, by studying several ethnic groups we would be able to examine the reported variation in the age of lactase downregulation between populations (Study I). In the next step, we extended the approach and investigated the mechanism of the developmental downregulation of lactase activity. For this, we obtained intestinal biopsies from children at various ages, and quantitated the relative lactase mRNA expressed from the C$_{-13910}$ and T$_{-13910}$ alleles, respectively (Study II).

1.1 Timing of lactase downregulation

The prevalence of the C/C$_{-13910}$ genotype of adult-type hypolactasia among the 329 children participating in Study I and representing three ethnic groups was as follows: 14.7% in the Finnish, 75.0% in the other Caucasians, and 95.4% in the African children. The prevalence figures are in the range we expected (50), and it is to be noted that all of the children from Somalia had the C/C$_{-13910}$ genotype. We additionally genotyped the G/A$_{-22018}$ variant (8) that shows a 98% association with the lactase persistence/non-persistence trait. In all cases except for two of the Finnish subjects, the G/G$_{-22018}$ genotype was present in children with the C/C$_{-13910}$ genotype. The younger of the two subjects with genotype combination C/C$_{-13910}$ and G/A$_{-22018}$, aged five, still had high lactase activity, but the other child, aged ten years, had low

lactase activity (5 U/g protein), giving further evidence for the conclusion that the G/A_{-22018} variant is only in linkage equilibrium with the causing variant (8).

In the combined study population representing various ethnic backgrounds and ages, the mean lactase activity in children with the C/C_{-13910} genotype was 13.8 U/ protein, in those with C/T_{-13910} genotype 33.1 U/g protein and in those with T/T_{-13910} genotype 50.4 U/g protein, demonstrating a trimodal distribution of lactase activities, as expected (7, 202-204). Among those with the C/C_{-13910} genotype aged > eight years, 93% had low lactase activity, here defined as lactase activity < 10 U/g protein or lactase to sucrase (L/S) ratio < 0.2. In all children with the C/C_{-13910} genotype who were > 12 years of age, lactase activity had decreased to < 10 U/g protein (Study I) (Figures 10 and 11). An analysis of disaccharidase activities in 39 random samples from adolescents aged 12-18 years genotyped for the C/T_{-13910} polymorphism who had undergone endoscopy at the Hospital for Children and Adolescents, University of Helsinki during a two-year period between 2003 to 2005 also showed that the subjects with the C/C_{-13910} genotype (n=5) presented with low lactase activity of < 10U/g protein whereas all those with genotypes C/T_{-13910} and T/T_{-13910} (n=34) had lactase activity ≥ 10 U/g protein (Rasinperä, unpublished data). The decline of lactase activity in Study I occurred somewhat earlier among the African children with the C/C_{-13910} genotype (Figure 11). Among those < five years of age, 67% of Africans compared to 25% of the Finnish children had lactase activity < 20 U/g protein ($p<0.04$). However, the disaccharidase activities in African children were overall lower than in Finnish children, as reported earlier (56), and the difference in the age of lactase downregulation was not significantly different when lactase activity < 20 U/g protein was used as the cut-off value. In the other Caucasian children with the C/C_{-13910} genotype, lactase activity had decreased to < 10 U/g protein in all children by six years of age.

Figure 10. Lactase activity in Finnish children with the C/C$_{-13910}$ genotype.

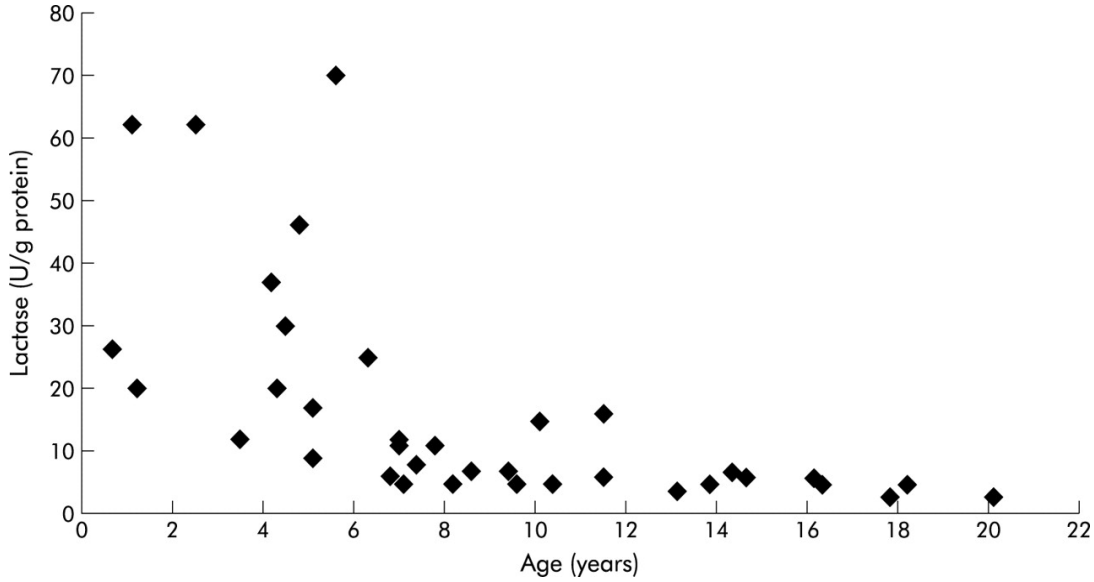
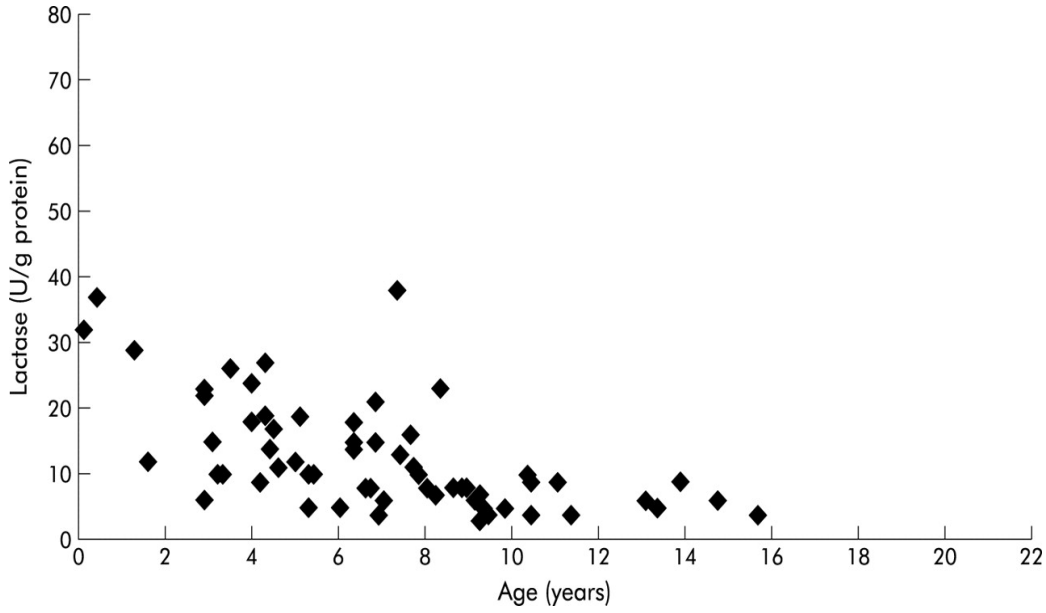


Figure 11. Lactase activity in the African children with the C/C$_{-13910}$ genotype.



Among the children with C/T$_{-13910}$ and T/T$_{-13910}$ genotypes defining lactase persistence, lactase activity was > 10 U/g protein in 98% of the cases. Of the four cases with lactase < 10 U/g, three had the C/T$_{-13910}$ genotype and one the T/T$_{-13910}$ genotype. One of these children was diagnosed to have congenital lactase deficiency (CLD) and another was carrier of a CLD mutation on the T$_{-13910}$ allele-carrying

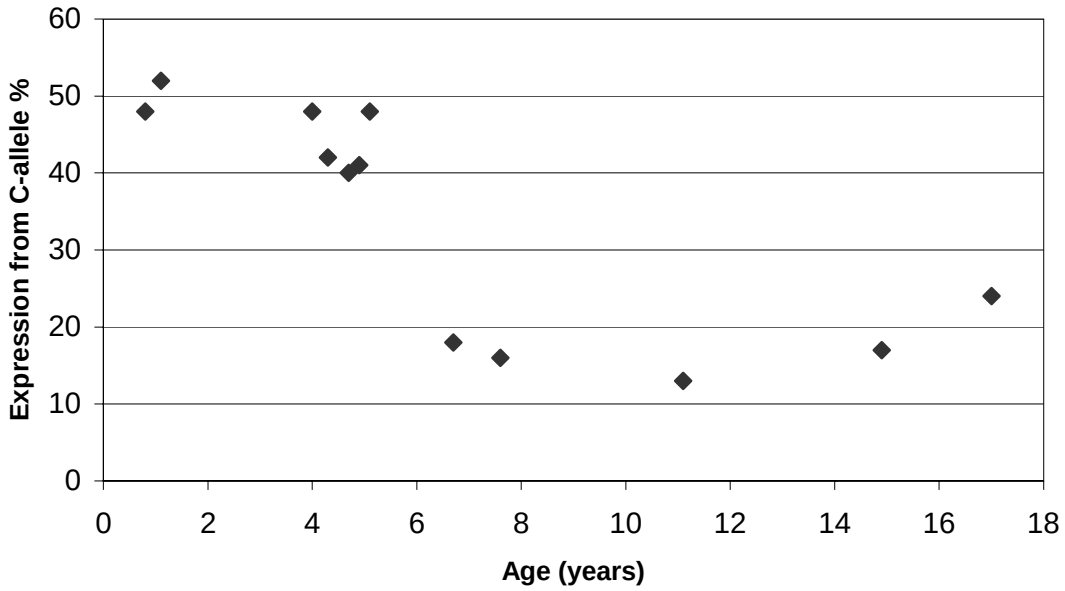
chromosome, and was confirmed to be a lactose malabsorber by LTT. The two other children had borderline lactase activities of 9 U/g protein with L/S ratios of 0.21 and 0.22, respectively; both had gastrointestinal symptoms when on a low lactose containing diet.

1.2 Mechanism of lactase downregulation

To further assess the role of the C_{-13910} allele in the downregulation of lactase activity (Study II), we collected an additional set of samples from children undergoing upper gastrointestinal endoscopy. An additional biopsy was taken from them, in order to measure the lactase expression at the mRNA level. We used G/A_{+593} polymorphism in exon 1 of the LCT gene as a marker of relative lactase mRNA expression. Consequently, we isolated total RNA from intestinal biopsy samples with verified disaccharidase activities in children heterozygous for this polymorphism (n=15). Twelve of them were heterozygous for the lactase persistent C/T_{-13910} genotype and three homozygous for the lactase non-persistent C/C_{-13910} genotype. The subjects with the C/T_{-13910} genotype had high lactase activity (mean activity of 47 U/g protein) except for one child who had a lactase activity of 6 U/g protein. This child was later shown to be a carrier of a CLD mutation. Of the three subjects with the C/C_{-13910} genotype, one, who was 23 years, had low lactase activity (6 U/g protein), a five-year old subject had high lactase activity (24 U/g protein) and from one subject lactase activity was not available.

In order to quantitate the relative expression levels of the C_{-13910} and T_{-13910} allele we used the allele-specific reverse transcription-polymerase chain reaction (RT-PCR) followed by solid-phase minisequencing. Our results showed that lactase mRNA was transcribed from both the C_{-13910} and T_{-13910} allele in children younger than five years in a 1:1 ratio. In those children over six years of age, the relative lactase mRNA expression from the C_{-13910} allele was reduced to 18% and 16% compared to that from the T_{-13910} allele (Figure 12).

Figure 12. Relation of age to the relative expression of LPH mRNA from the C_{-13910} compared to that from the T_{-13910} allele.



1.3 Discussion

These two studies are the first to explore the timing of downregulation of lactase activity in children genetically tested for the C/T_{-13910} variant associated with lactase persistence/non-persistence. Our findings demonstrate, both at the protein and at the mRNA level, a causative role for the C_{-13910} allele in the development of adult-type hypolactasia in subjects with the C/C_{-13910} genotype. To conclude, 1) we have observed a marked imbalance in relative lactase mRNA expression levels from the C_{-13910} compared to the T_{-13910} allele in children aged > five years. Moreover, the expression of the transcribed lactase mRNA from the C_{-13910} allele (Study II) declines around the same age when the developmental downregulation of lactase activity occurred (Study I). 2) The prevalence of the C/C_{-13910} genotype in the ethnic groups (Study I) was well in agreement with the reported prevalence of lactose malabsorption in the Finnish and African populations (50), and 3) the trimodal distribution of lactase enzyme activity according to the C/T_{-13910} genotype groups (Studies I and II), further demonstrates an association of the C/T_{-13910} variant with the lactase persistence/non-persistence trait.

The age of onset of adult-type hypolactasia has been reported to vary between populations (50). In the Finnish population adult-type hypolactasia has been reported to manifest up to 20 years of age (62, 63). Our study, however, suggests that lactase activity is indeed downregulated in Finnish children with the C/C$_{-13910}$ genotype by the age of 12 years. Studies on the age of onset of adult-type hypolactasia in subjects of African origin are scarce in number. Welsh, et al, have reported that adult-type hypolactasia develops after the age of three years in black children; the exact ethnic background of the subjects, however, was not reported (55). In another study the prevalence of lactose intolerance based on LTT in black children living in Boston, USA, was reported to have an increasing gradient from 11% at the age of 4-5 years to 72% in subjects aged 8-9 years (254). We noticed (Study I) that the difference in the age at which lactase activity is downregulated between the two genetically diverse populations, Finnish and Somali, is somewhat smaller than previously reported in the literature (50, 55, 254). Lactase mRNA expression was only examined in Finnish children (Study II). We did not observe any difference in the relative expression levels from the C$_{-13910}$ and T$_{-13910}$ alleles in children younger than four to five years of age. Variations in the levels of lactase enzyme activity and L/S ratio may indicate differences in other regulatory factors (36, 89, 91, 92, 94, 211, 255) or posttranscriptional modifications (73). Naturally, the level of lactase expressed as U/g protein, is also to a great extent influenced by technical variations and the site where the biopsy specimen is obtained; in our studies this bias, however, can be considered minimal since all endoscopies were carried out at the same hospital and by experienced surgeons. The regulatory mechanisms underlying the reported ethnic differences in the timing of downregulation of lactase activity, however, are so far poorly understood.

Our results (Study II) about the transcriptional regulation of the lactase gene support the earlier findings by researchers in other laboratories (69-74). Our own laboratory has showed that the persistent T$_{-13910}$ allele represents a mean 92% of the expressed lactase mRNA in adults heterozygous for the C/T$_{-13910}$ genotype (209). Previously, quantitation of allele-specific expression of lactase mRNA by a different method from ours in 15 lactase persistent children heterozygous for the same polymorphism G/A$_{+593}$ in exon 1 of the lactase gene as analysed here, has shown equal expression in fetal samples and in the majority of those < 16 months (72). However, the method

used in those cases was based on detection and quantitation of band intensity on autoradiographs, and it differs from our method in its accuracy.

Recent studies assessing the functional role of the C/T$_{-13910}$ polymorphism have demonstrated differential regulation of lactase promoter activity and binding capacity for the nuclear proteins in electromobility shift assay (EMSA) for the C$_{-13910}$ and T$_{-13910}$ allele (96, 210). In EMSA, the T$_{-13910}$ probe has been observed to interact more strongly and to a more abundant specific DNA/protein complex in comparison with the C$_{-13910}$ probe (96, 210). In another current study (211), DNA affinity purification resulted in isolation of the Oct-1 transcription factor with glyceraldehyde-3-phosphate (GADPH) bound directly to the T$_{-13910}$ variant site. Co-expression of Oct-1 binding the T$_{-13910}$ enhancer region with HNF-1α (transcription factor with a binding site at the proximal lactase promoter) increased the effect of the T$_{-13910}$ enhancer 133-fold over the proximal promoter activity. Oct-1 showed lower affinity to the C$_{-13910}$ sequence, and the Oct-1/HNF-1α co-expression increased the expression less: 112-fold. Binding sites for other transcription factors with importance to intestinal gene expression, namely GATA-6, HNF4α, Fox/HNF3α and Cdx-2, were detected by DNase footprint and supershift analyses and characterized within the –13910 region. Mutations in any of above-mentioned transcription factor binding sites were observed to abolish the activity of the –13910 enhancer, demonstrating that the presence of all of the above mentioned binding sites is necessary for the –13910 region to possess enhancer activity. It was also established that the –13910 enhancer has a preference for the lactase promoter since it did not activate the MCM6 promoter in a transfection experiment (211).

Based on these lines of evidence the –13910 region contains a strong transcriptional enhancer and substitution of cytosine to thymidine at –13910 site seems to have resulted in an increased activity of the enhancer and created a stronger Oct-1 binding site. At the time after weaning (mammals) or during childhood (humans) when lactase activity should decline in adult-type hypolactasia, the presence of Oct-1 at the T$_{-13910}$ enhancer may lead to either increased recruitment of activators of transcription or prevention of action of a repressor. This, in theory, compensates for the weak LCT promoter and ensures an active LCT gene throughout life. Lewinsky, et al (211), even speculate that since Oct-1 has been reported to recruit chromatin modifying co-factors

(256), it might be possible that the binding of Oct-1 to the T_{-13910} variant region *in vivo* would induce changes in the chromatin structure around the LCT gene, thus enhancing or silencing LCT expression. Despite these speculations, the detailed spatiotemporal pattern of specific protein-protein interactions between the –13910 enhancer region and the lactase promoter in inducing lactase non-persistence/persistence phenotypes during childhood still remains to be characterized.

2. GENETIC TEST OF THE C/T_{-13910} VARIANT IN DIAGNOSIS OF ADULT-TYPE HYPOLACTASIA

The applicability of the genetic test of the C/T_{-13910} polymorphism in diagnosis of adult-type hypolactasia was assessed in the pediatric population (Study I) and in the adult population (Study IV). Our reference to the molecular diagnosis was the measurement of lactase activity and L/S ratio in the small intestinal mucosa (Study I) or in the large-scale study questionnaire-based (Study IV). Based on a questionnaire, the general attitudes towards the genetic test of adult-type hypolactasia and the probable effect of the test result on milk consumption were determined among 163 children and their families (Study III).

2.1 Genetic test of adult-type hypolactasia in children

Genotyping of the C/T_{-13910} variant associated with lactase persistence/non-persistence status in > 300 children, with known disaccharidase activities, using a lactase activity of < 10U/g protein to be indicative of adult-type hypolactasia, the sensitivity of the genetic test (i.e. the probability of the C/C_{-13910} genotype in genetic test when the person has low lactase activity) was 93%. The specificity (i.e. the probability of genotype C/T_{-13910} or T/T_{-13910} being found in a genetic test when the person has high lactase activity) was 80%. However, adult-type hypolactasia is a trait that develops during childhood, thus the specificity of the genetic test in children > eight years of age was 97% and in those > 12 years of age 100%. The positive predictive value (i.e. the probability that a person with the genotype C/C_{-13910} has low lactase activity) had an increasing gradient, ranging from 10% in children < five years of age to 65% in the age group of six-11 years and 100% in children aged > 12 years.

2.2 Comparison of molecular diagnosis to LTT results in adults

We asked the participants (n=1900) using questionnaires (study IV) about possible gastrointestinal symptoms and whether they had previous diagnosis of adult-type hypolactasia diagnosed by the lactose tolerance test (LTT). In this study with an extremely high response rate of 99%, among the participants with the C/C_{-13910} genotype, 19% reported a positive LTT. It is of interest that of those with the C/T_{-13910} genotype 10% (n=89) and those with the T/T_{-13910} genotype 14% (n=91) also reported being diagnosed as lactose malabsorbers by LTT. For seven of these in a total of 190 subjects with genotypes C/T_{-13910} or T/T_{-13910} suggesting lactase persistence, the explanation for positive LTT could be a previously diagnosed celiac diasease. In addition, positive screening tests based on elevated level of transglutaminase antibodies in serum were observed in two other subjects. (Tikkakoski, submitted).

2.3 Acceptance and implications of the genetic test

We determined the genotypes for the C/T_{-13910} variant in a group of 172 children participating in a follow-up study on cow's milk allergy and their controls (Study III) (222). The acceptance of the genetic testing was very good as only two families refused to participate. The families of the children also filled in a questionnaire concerning gastrointestinal symptoms, including one question on the probable effect of the result of the genetic test on their milk consumption. Of all the families, 13% reported that the test result would have an effect on their milk consumption: among those with the C/C_{-13910} genotype, one third of the children reported a probable effect on their milk consumption. Of those children with the genotypes underlying lactase persistence, 10% declared they would from now on consume more lactose containing milk.

2.4 Discussion

The genetic test of the C/T_{-13910} variant is in diagnostic use in several laboratories in Finland and Sweden. Several recent publications report technical advances providing more rapid and labor-saving means for performing the genetic test for the C/T_{-13910} variant (215, 216, 257, 258). The C/T_{-13910} polymorphism, however, has been

suggested not to explain the lactase persistence/non-persistence trait in populations in Sub-Saharan Africa and therefore it has been stated that the genetic test of the C/T$_{-13910}$ polymorphism is premature and should not be used on African and other non-northern European populations (218).

Our results showed an excellent sensitivity and specificity for the genetic test in Finnish and Somali subjects by the age of 8-12 years. The genetic test of the C/T$_{-13910}$ variant has been compared with the results of breath hydrogen test (BHT) and lactose tolerance test (LTT) in some recent studies (213, 215, 216, 258). These studies have chiefly suggested a relatively good correlation with the indirect tests in Caucasian populations. In an Austrian study the sensitivity and specificity of the genetic test were 75% and 99%, respectively, compared with the BHT (213), and in Swedish subjects the genetic test and LTT showed concordant results in 94% of the subjects (216). In most cases, the discrepant results have been obtained in subjects with the heterozygous C/T$_{-13910}$ genotype (213, 215, 216). However, studies comparing the C/T$_{-13910}$ genotyping results to those of the indirect methods, with varying sensitivity and specificity (4), have their limitations. It should be kept in mind that the interpretation of the results obtained by indirect methods always depends on the cut-off level chosen. Based on a Finnish study, the practice of carrying out lactose tolerance tests in Finnish health care centers varies widely with respect to the reference values as well as the follow-up of the symptoms (259). Furthermore, the indirect test methods cannot rule out secondary lactase deficiency.

It is obvious that the genetic test of adult-type hypolactasia is most useful in populations with relatively high prevalence of adult-type hypolactasia and in which the consumption of dairy products is common. In some Western populations, including those of Northern European countries, such as Sweden and Denmark, the prevalence of adult-type hypolactasia is low (50). However, in many of those populations there are today high numbers of immigrants from populations with extremely high prevalences of adult-type hypolactasia, such as the observed 100% prevalence of the C/C$_{-13910}$ genotype in Somali children living in Finland (Study I). Thus, as the immigrants change their traditional diet to the lactose-containing diet of the new country and gastrointestinal symptoms appear (165), the diagnostic value of

the genetic test of adult-type hypolactasia can be assumed to become of more importance in these countries with mixed populations, too.

Importantly, the genetic testing should be applied only after the age when the downregulation of lactase activity is complete, this being in Finnish children after 8-12 years of age. There is concern that the molecular determination of the genetic predisposition to adult-type hypolactasia in children, at a time when lactase activity still is high or there are no symptoms of lactose intolerance, might lead to potentially harmful reduction in dairy product intake. Reduced calcium intake has been shown to contribute to reduced bone mineral density and osteoporosis (131). In general, the general recommendation is that predictive genetic testing of children should not be carried out unless there is an effective therapeutic intervention that can be offered (260, 261). In the case of lactose intolerance the treatment is easily performed by changes in diet, especially in countries such as Finland, which have a good availability of low-lactose dairy products as well as lactose-free milk, and with supplementation of calcium if needed. Furthermore, pharmaceutical preparations of lactase enzyme are available in most countries. However, one of the pitfalls of the genetic test of the C/T_{-13910} polymorphism is that the genotype C/C_{-13910}, predictive of adult-type hypolactasia, does not tell either the age at which the symptoms will develop, or if any symptoms will develop or be experienced at all. Furthermore, the amount of lactose tolerated by the subjects with the C/C_{-13910} genotype is not known. Based on our results, one third of the children with the genotype C/C_{-13910} reported a probable effect on their milk consumption, whereas 10% of those with the C/T_{-13910} and T/T_{-13910} genotypes reported that the test result was helpful in avoiding unnecessary restrictions in their consumption of lactose containing products (Study IV).

In clinical practice, the role of the genetic test has been suggested to be limited to ruling out adult-type hypolactasia as a cause of intolerance symptoms (262). Furthermore, concern that the gastrointestinal symptoms of a patient with genotype predisposing to adult-type hypolactasia might be attributed merely to symptoms of lactose intolerance, delaying or preventing diagnosis of more serious causes of lactose intolerance due to secondary lactose intolerance, has been presented (262). Taken all this into consideration when interpreting the gene test results, it is still obvious that as

a first-stage screening method for adult-type hypolactasia, the advantages of the genetic test in comparison to the biochemical methods such as LTT and BHT are apparent, both for the patients and the laboratory personnel. The genotype of lactase persistence/non-persistence can be determined from one blood sample taken without fasting using semi-automated analysis, saving time and costs to health care services. Furthermore, the test is also more comfortable for the patients; it needs to be done only once in a lifetime and the interpretation of the test result is unequivocal (263). To summarize, the genetic test of adult-type hypolactasia has an excellent specificity and sensitivity in excluding primary hypolactasia as a cause of gastrointestinal symptoms in subjects of Caucasian origin. Further studies are needed to answer the question whether there exists other polymorphisms in populations where the T_{-13910} allele is underpresented or not yet studied (218).

3. PREVALENCE OF GASTROINTESTINAL (GI) SYMPTOMS AND CONSUMPTION OF MILK PRODUCTS IN THE FINNISH POPULATION IN RELATION TO THE GENOTYPE OF ADULT-TYPE HYPOLACTASIA

Gastrointestinal symptoms and consumption of milk products were assessed by a questionnaire-based approach in Finnish children (Studies I and III), and in Finnish adults (Study IV) with a more comprehensive questionnaire covering questions on the specific symptoms, their frequency and relation to meals, as well as consumption of particular dairy products, among others. In these studies we genotyped a total of 2118 subjects completing the questionnaires for the C/T_{-13910} genotype, and thus were able to examine the reported milk related gastrointestinal symptoms and milk consumption based on the subjects' molecularly defined lactase persistence/non-persistence status.

3.1 Milk related symptoms and milk consumption in Finnish children

In the first study (Study I), 22% of the Finnish children or their families completed a questionnaire about milk consumption and possible milk related symptoms. The response rate remained low, because we started the collection of the milk related data late during the study. Later on, 162 children with a history of cow's milk allergy and

their controls filled in the questionnaires about milk consumption and specific gastrointestinal symptoms (Study III). The questionnaire used in this study was somewhat more comprehensive compared to the one used in Study I; thus the results of the questionnaires cannot be directly compared.

Among participants in Study I, 35% of the children reported symptoms from milk products; however, no significant difference between symptoms reported by children with the C/C$_{-13910}$ genotype and those with the genotypes C/T$_{-13910}$ and T/T$_{-13910}$ associated with lactase persistence was observed. In Study III, abdominal symptoms during a one-week period were reported by 23% of the children in total: by one third of those with the C/C$_{-13910}$ genotype and by 27% and 15% of those with the C/T$_{-13910}$ and T/T$_{-13910}$ genotypes, respectively ($p=0.05$; C/C$_{-13910}$ compared to T/T$_{-13910}$ genotype). Among the specific gastrointestinal symptoms, such as flatulence, loose stools, and constipation, experienced by the children, flatulence was the only symptom that differed according to the C/T$_{-13910}$ genotype. Flatulence was reported by 19% of those with C/C$_{-13910}$ genotype compared to 12% and 5% of those with C/T$_{-13910}$ and T/T$_{-13910}$ genotypes, respectively ($p<0.04$; C/C$_{-13910}$ compared to T/T$_{-13910}$ genotype).

A significant difference in milk drinking habits between the C/C$_{-13910}$ and the non-C/C$_{-13910}$ genotype groups was observed in both Studies I and III. In Study I, 57% of the children with the C/C$_{-13910}$ genotype associated with adult-type hypolactasia reported that they never drank milk (Table 6). In Study III, 44% of the children with the C/C$_{-13910}$ genotype reported drinking < one dl of milk per day. In Study I, only 3% of those with the C/T$_{-13910}$ or T/T$_{-13910}$ genotypes reported never drinking milk ($p<0.001$ compared to those with C/C$_{-13910}$ genotype) and in Study III, 20% of those with C/T$_{-13910}$ and T/T$_{-13910}$ genotypes reported drinking < one dl milk daily ($p<0.02$ compared to those with C/C$_{-13910}$ genotype). It should, however, be noted that in 21% of the children reporting drinking < one dl of milk in Study III, despite their lactase persistence/non-persistence genotype, cow's milk allergy (CMA) was confirmed in a challenge test (each of them had a previous diagnosis of CMA). Notably, in Study III, 93% of the children with C/C$_{-13910}$ genotype, anyhow, reported consuming milk products; in 36% of these cases a low lactose containing diet was reported. No

correlation with the C/C$_{-13910}$ genotype of adult-type hypolactasia to the presence of CMA was observed.

3.2 GI symptoms and milk consumption among Finnish adults

During a three-month period in spring 2004, in total 99% of the adults participating in the study for genotyping the C/T$_{-13910}$ variant in primary health care filled in the questionnaire. Among adults, 80% reported having experienced gastrointestinal (GI) symptoms during the previous three months, 84% of those with the C/C$_{-13910}$ genotype and 79% and 78% of those with the C/T$_{-13910}$ ($p<0.05$) and T/T$_{-13910}$ ($p<0.05$) genotypes, respectively. Flatulence was the only symptom that the subjects with the genotype C/C$_{-13910}$ reported more frequently compared to the subjects with the genotypes C/T$_{-13910}$ ($p=0.06$) and T/T$_{-13910}$ ($p<0.05$). Those with the C/C$_{-13910}$ genotype reported GI symptoms somewhat but not significantly more frequently during one week. It is of interest that among the participants of Study IV who attended primary health care because of gastrointestinal symptoms, the C/C$_{-13910}$ genotype was observed in 24% of the subjects, that is significantly more often than the observed 18% prevalence of the C/C$_{-13910}$ genotype in the total study population ($p<0.05$).

In Study IV, 82% of Finnish adults with genotype C/C$_{-13910}$ suggestive of adult-type hypolactasia reported that they did not drink milk with meals. The figure is significantly higher than the approximately 60% of those with genotypes C/T$_{-13910}$ and T/T$_{-13910}$, respectively, who report not drinking milk with meals ($p<0.0001$) (Table 6). Among the subjects drinking milk with meals, those with the C/C$_{-13910}$ genotype reported milk related symptoms somewhat but not significantly more often than the lactase persistent subjects. Only a minority of the subjects with the C/C$_{-13910}$ genotype drank milk with meals and reported no symptoms from it. The estimation of the percentage of milk drinking but asymptomatic subjects with the C/C$_{-13910}$ genotype in our study, however, is difficult since one third of the milk-drinking subjects with C/C$_{-13910}$ genotype did not answer the question on the symptoms related to milk. Cheese, on the other hand, was reported to cause symptoms for 17% of those with the C/C$_{-13910}$ genotype compared to 10% and 9% of those with the C/T$_{-13910}$ and T/T$_{-13910}$ genotypes, respectively ($p<0.05$).

Table 6. Milk drinking by Finnish children and adults with regard to the C/T_{-13910} genotype. The data for children not drinking milk is combined from Studies I (subjects reporting never drinking milk) and III (subjects reporting drinking < one dl of milk/day).

	C/C_{-13910}		C/T_{-13910}		T/T_{-13910}	
	Children	Adults	Children	Adults	Children	Adults
Milk	53% (17/32)	18% (60/334)	88% (96/109)	38% (333/887)	83% (63/76)	36% (236/647)
No milk	47% (15/32)	82% (274/334)	12% (13/109)	62% (554/887)	17% (13/76)	64% (411/647)

3.3 Discussion

Our results (Study IV) showed that gastrointestinal (GI) symptoms are very common among the Finnish working age population. In a recent Finnish population-based study, 10% in an unselected cohort of young adults reported major GI-complaints, and more than third reported minor GI-complaints. Symptoms related to milk were reported by every fourth of the subjects; 6% of the subjects reported symptoms related to milk with low-lactose content, too (162). In a Finnish population-based study on school-aged children, 10% of the children reported GI symptoms related to milk; in 8% of the children the symptoms were compatible with lactose intolerance (264). These figures are to some extent lower than in our studies on Finnish children; however it should be noted that our study population was somewhat pre-selected as the participants were either a subpopulation of children attending endoscopy because of GI complaints (Study I), or attending a follow-up study because of cow's milk allergy and their controls (Study III). In any case, all these studies, demonstrate that unspecific GI complaints, especially those related to milk, are very common among Finnish children and adults.

Finland has the highest consumption of milk products of any country (235). Study IV, though, demonstrated that only one third of Finnish adults reports drinking milk with

meals. In our study population 69% of young men reported drinking milk daily, this group being the most frequent milk drinkers. Among middle-aged women, only 26% reported drinking milk daily. Men, in general drank milk more than women, in all age groups studied (unpublished data). It is possible that this is due to factors simply related to a dislike of the taste of milk. Also, it can be speculated to be a combination of higher prevalence of gastrointestinal disorders in women, in general (158), and the common subjective feeling of milk often underlying them, as observed in Study IV. In Study I, not taking the C/T_{-13910} genotypes of the subjects into consideration, 55% of the Finnish children participating reported drinking > two dl of milk/day, 37% reported drinking one -two dl of milk/day and 8% not drinking milk at all. In Study III, among the eight - nine years old children with a previous diagnosis of cow's milk allergy and their controls, 90% reported consuming milk products, although 24% reported drinking < 1 dl of milk/day. Thus, based on our studies < 10% of the Finnish children report drinking very little or no milk at all. To conclude, these data demonstrate that drinking milk with meals is common among Finnish children, but an increasing gradient in those excluding milk products is observed with increasing age among Finnish adults.

A variety of abdominal symptoms are frequently attributed to lactose intolerance (265). In well-controlled studies, however, GI symptoms have been shown to occur independent of lactose intake (14). It has been observed that among those with self-reported lactose intolerance only a proportion, in most studies < 70% of the subjects, are true lactose maldigesters (3, 162, 265-267). In our studies, GI symptoms were observed to occur frequently among both subjects with molecularly defined adult-type hypolactasia as well as the participants with lactase persistence. However, we observed that those adults with the genotype associated with adult-type hypolactasia used primary health care services significantly more often due to GI complaints than the lactase persistent subjects. In all the studies (I, III, and IV), subjects with the C/C_{-13910} genotype of adult-type hypolactasia had self-restricted their milk consumption. However, some other component of milk than lactose, if any, seems to underlie the GI symptoms related to milk reported by the subjects with the C/T_{-13910} and T/T_{-13910} genotypes.

The amount of lactose tolerated in hypolactasia depends on several factors: the lactase activity in the small intestine, the composition of the colonic microbiota, the composition of the food in which lactose is consumed and the individual sensitivity to the stretching of the intestinal wall, among others (109). Adaptation to lactose consumption has been speculated to play some role as well (128, 268, 269). Taken these facts into consideration, it comes clear that it is very difficult to examine the level of lactase activity that would be needed in order to prevent lactose intolerance. We could not find any significant difference in symptoms from milk except for flatulence (Study III) between those children with the C/C$_{-13910}$ genotype and those with genotypes suggesting lactase persistence. Secondly, we did not have the exact amounts of milk (lactose) consumed by the children available. Finally, our study population included children at various ages around the age at which lactase enzyme activity is downregulated and as only 28% of them were interviewed for the abdominal symptoms, the number of symptomatic children with the C/C$_{-13910}$ genotype and low lactase activity, unfortunately, was too small for any conclusions to be drawn for this complex issue.

4. THE ROLE OF ADULT-TYPE HYPOLACTASIA IN THE DEVELOPMENT OF COLORECTAL CARCINOMA

Diet has an important role in modification of the risk of colorectal cancer (CRC) (270). The role of dairy products in pathogenesis of CRC has been extensively studied, however, it has remained unclear (229, 233, 234, 236, 237, 239). Using the C/T$_{-13910}$ variant associated with lactase persistence/non-persistence trait as a molecular marker, we conducted a large-scale study on a possible association between adult-type hypolactasia and CRC (Study V).

4.1 Results

We observed that the C/C$_{-13910}$ genotype associated with adult-type hypolactasia was found significantly more often among the Finnish subjects with colorectal carcinoma than among their controls ($p<0.02$), with an odds ratio (OR) of 1.40 (1.07-1.85) for having the genotype C/C$_{-13910}$ and colorectal cancer, in comparison to the other

genotypes, C/T$_{-13910}$ and T/T$_{-13910}$. We had clinical data, including age at the time of diagnosis, tumour histology, stage and site of the tumour among others, for two of the three subpopulations of Finnish subjects with CRC available, in total for 92% of the cases. We, however, did not observe any significant differences when comparing the clinical characteristics of the patients with the C/C$_{-13910}$ genotype and the ones with the C/T$_{-13910}$ and T/T$_{-13910}$ genotypes.

Among the British subjects, the overall frequency of the C/C$_{-13910}$ genotype was 9.0%, somewhat higher than the earlier reported frequency of adult-type hypolactasia in the United Kingdom (203, 271). Among the Spanish subjects of Catalonian origin, the total C/C$_{-13910}$ genotype frequency was 34.6%. There was no difference in the lactase persistence/non-persistence genotype frequencies in British or Catalonian cases and controls. For the British and Spanish subjects, no clinical data was available.

4.2 Discussion

The consumption of dairy products has been suggested in several studies to be associated with a modest reduction in the risk of colorectal cancer (229, 233, 234). Finns have been observed to have the highest intake of milk and dairy products in the world, higher than that in the UK and Spanish populations (235). The dairy consumption of the subjects participating in this study, unfortunately, was not available. However, in our other studies on the Finnish population (Studies I, III, and IV) we observed that those with the C/C$_{-13910}$ genotype associated with low lactase activity drink significantly less milk than those with genotypes C/T$_{-13910}$ and T/T$_{-13910}$ and high lactase activity. It can be speculated that one reason affecting the elevated risk of colorectal cancer among those with the C/C$_{-13910}$ genotype, could be their lower consumption of milk products shown to be protective against CRC. The slightly elevated risk observed only among the Finnish but not in the British and Spanish populations, on the other hand, could be a consequence of the risk being detected only in the populations where milk products have such an important role in the diet as in Finland, or where the differences in milk consumption among the C/T$_{-13910}$ genotype groups are as evident as in Finland.

Ingestion of lactose has been shown to alter the populations of the intestinal microbiota (272). In subjects with adult-type hypolactasia, lactose consumed has been suggested to act as a prebiotic (246, 273), i.e. as a food ingredient that is not digested, but that can indirectly stimulate growth and/or activity of lactic acid producing bacteria in the colon. In this sense, it would be assumed that subjects with adult-type hypolactasia consuming lactose regularly would be protected from colorectal cancer. Montalto, et al, have suggested inspired from our study that, in order to be able to assess the possible supplementary protective effect of lactose as a prebiotic, the population with adult-type hypolactasia should be stratified into two groups, the symptomatic and the asymptomatic subjects (274). Furthermore, Montalto et al, suggest that the risk in subjects consuming low lactose milk should be determined (274).

As mentioned, the limitation of our study is the lack of the milk consumption data from the study participants. Without the data it is difficult to make any further conclusions on the harmfulness of lactose as such to those with the C/C_{-13910} genotype since the increased risk could also be caused by a lower intake of dairy products in comparison to those with C/T_{-13910} or T/T_{-13910} genotype. To summarize, this study is the first to have assessed the role of lactase activity in the development of colorectal cancer on this scale. The results of this study are new and exciting, but further studies are needed to confirm them. For this, a study assessing the role of consumption of milk products in subjects with the exact clinical course of colorectal cancer and molecularly defined lactase persistence/non-persistence status is underway.

CONCLUSIONS AND FUTURE ASPECTS

The identification of the C/T$_{-13910}$ polymorphism associated with lactase persistence/non-persistence trait in 2002 opened new avenues for basic and applied research into adult-type hypolactasia. The molecular marker facilitated studies on the timing and mechanism of the developmental downregulation of lactase activity. In this study we have demonstrated an excellent correlation between low lactase activity and the C/C$_{-13910}$ genotype in all subjects > 12 years of age, irrespective of their ethnicity. Furthermore, we have demonstrated an increasing imbalance in the relative lactase mRNA expression from the C$_{-13910}$ and T$_{-13910}$ alleles in Finnish children beginning from five years of age, supporting causative role of this variant in adult-type hypolactasia. The genetic test for adult-type hypolactasia has been introduced into routine diagnostics as an exclusion test for adult-type hypolactasia as a cause of abdominal symptoms. In this study the test showed a specificity of 100% in the Finnish children and adolescents > 12 years of age.

Milk is an important component of the Northern European diet. In this work we, however, show that a significant percentage of Finnish children and adults report gastrointestinal symptoms related to milk. Comparison of milk consumption and milk related symptoms with regard to the C/T$_{-13910}$ genotype of the participants revealed that Finnish children and adults with the C/C$_{-13910}$ genotype associated with low lactase activity drink significantly less milk and report flatulence significantly more often than those with genotypes associated with lactase persistence. A proportion of subjects with the C/C$_{-13910}$ genotype, nevertheless, reported drinking milk without experiencing any symptoms. In an association study an increased risk of colorectal cancer was observed among those with molecular diagnosis of adult-type hypolactasia. It, however, remained to be clarified whether the increased risk observed in the Finnish population could have been caused by lactose or decreased intake of dairy products in these subjects.

Lactose intolerance is a complex phenotype. The genetic test of the C/T$_{-13910}$ variant cannot exclude lactose intolerance; however, it can exclude primary adult-type hypolactasia underlying the symptoms. A predictive genetic test for adult-type

hypolactasia is of no value in children without symptoms from milk, at least as far as lactose has not been demonstrated to be harmful for those with the C/C_{-13910} genotype. In future, the role of the C/T_{-13910} SNP as the causing variant of adult-type hypolactasia in populations, including those of sub-Saharan Africa needs to be reconsidered. New insights into the molecular mechanisms underlying developmental downregulation of lactase activity have recently been obtained, however, the exact functional role of the C/T_{-13910} variant and the components involved remain to be clarified.

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